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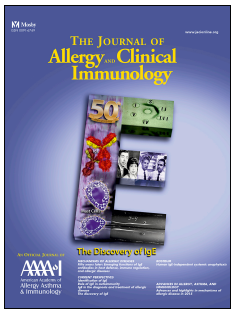

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Extracellular eosinophilic traps in association with *Staphylococcus aureus* at the site of epithelial barrier defects in severe airway inflammation

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Abstract (250 words)

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a Th2-biased eosinophilic inflammation. Eosinophils have been shown to generate so-called extracellular eosinophilic traps (EETs) under similar pathological conditions.

Objective: Our aim was to investigate a possible link between EET formation and the presence of *Staphylococcus aureus*, an organism frequently colonizing the upper airways, at the human mucosal site of the disease.

Methods: Tissue slides were investigated for the presence of EETs and *S. aureus*, using immunofluorescent staining and PNA-fish assay respectively. An *ex vivo* human mucosal disease tissue model was used for artificial infection with *S. aureus*. Cell markers were analyzed using immunohistochemistry, luminex Multiplex assay, ELISA, PCR, immunobloting and linked to the presence of EETs.

Results: About 8.8 ± 4.8 % of the infiltrating eosinophils exhibited EETs in patient's nasal polyp tissues. The formation of EETs was associated with increased IL-5 ($p < 0.05$) and periostin ($p < 0.05$) tissue levels, and colonization with *S. aureus (p < 0.05)*. Using an *ex vivo* human mucosal disease tissue model, EET formation was induced (4.2 ± 0.9 fold) upon exposure to *S. aureus*, but not to *S. epidermidis*. Eosinophils were shown to migrate ($p < 0.01$) towards *S. aureus* and entrap the bacteria both inside and outside the mucosal tissue. Blocking NAPDH oxidase activity, led to a complete inhibition ($p < 0.05$) of EET formation by *S. aureus.*

Conclusion: Eosinophils are likely to be specifically recruited to and form EETs at sites of airway epithelial damage to protect the host from infections with *S. aureus* and possibly other microorganisms in CRSwNP.

Capsule Summary

Eosinophils in nasal polyp tissue are likely to be recruited to sites of airway epithelial damage to protect the host against *S. aureus* infections by forming EETs.

Key Messages

- EETs are formed in nasal polyp tissue, mainly at subepithelial sites with epithelial barrier defects, and are associated with increased IL-5 tissue levels and *S. aureus colonization*.
- EET formation is induced in nasal polyp tissue, upon exposure to *S. aureus*, but not to *S. epidermidis*.
- Eosinophils migrate towards *S. aureus* and entrap the bacteria both inside and outside the mucosal tissue.
- Reactive oxygen species are involved in this rapid process.

List of abbreviations

AD : atopic dermatitis

C5a : Complement factor 5a

CRSwNP : Chronic rhinosinusitis with nasal polyps

DAPI : 4',6-diamidino-2-phenylindole

DNA : Deoxyribonucleic acid

DPI : diphenyleneiodionium

E. coli : Escherichia Coli

EETs: extracellular eosinophilic traps

IFN : interferon

IL-5 : Interleukin

IT : inferior turbinate

lfTSLP : long form TSLP

LPS : lipopolysachariden

MBP : Major Basic Protein

MGG : May-Grünwald-Giemsa staining

ROS : reactive oxygen species

S. aureus : Staphylococcus aureus

S. epidermidis : Staphylococcus epidermidis

sfTSLP : short form TSLP

TCM : Tissue culture medium

Th2 : T-helper 2

TSLP : Thymic stromal lymphopoietin

1. Introduction

Asthma, allergy, aspirin hypersensitivity and chronic rhinosinusitis with nasal polyps (CRSwNP) have all been characterized as Th2-biased eosinophilic inflammations and often represent comorbid diseases. [1-3] Nasal polyp tissue of Caucasian patients usually shows high levels of interleukin IL-5 and eosinophil-related chemokines.[4] Together, these cytokines seem to orchestrate the chemotaxis, activation and survival of eosinophils.[1,5,6] Tissue eosinophilia increases the likelihood of recurrent disease and comorbid asthma in CRSwNP patients, indicating that eosinophils play a central role in the pathology of CRSwNP.[7] However, their role in airway disease and their contribution to the inflammation is not fully understood.

Eosinophils have cytotoxic functions and are involved in both the innate and adaptive immune responses; they have been correlated with epithelial damage via the release of major basic protein (MBP).[8] In addition, activated eosinophils can contribute to antibacterial defense by releasing mitochondrial DNA in association with granule proteins.[9,10] *In vitro*, these so-called eosinophil extracellular traps (EETs) are able to bind and kill bacteria like *S. aureus*, *S. epidermidis* and *E. coli*.[10,11] Here we hypothesized that EETs are present and are activated in nasal polyp tissue by *S. aureus.* As a consequence, these findings could provide further insights and understanding of the underlying causative mechanisms that lead to eosinophilic nasal polyps.

In vitro co-culture with eosinophils was sufficient to evoke EETs in the case of *S. aureus*, an additional stimulus with thymic stromal lymphopoietin (TSLP) was required for *S. epidermidis* to generate traps *in vitro,* suggesting differing responses of eosinophils according to the type of bacteria.[10,11] Enzymatic digestion of extracellular DNA obviates the capacity of EETs for killing bacteria. This indicates that, despite the fact that DNA itself has no antibacterial effect, EETs need both intact DNA and granule proteins to orchestrate this function.[12] The generation of EETs is considered to be an active process, not associated with cell death.[10,12] *In vitro,* EET formation is stimulated by priming eosinophils with IL-5, and interferon (IFN)-ɣ or adhesion molecules followed by activation with complement factor 5a (C5a), LPS, TSLP, and eotaxin.[11,12]

In addition to its association with eosinophilic inflammation, CRSwNP is also associated with a strong colonization by *S. aureus*.[13] Interestingly, eosinophils were recently reported to generate EETs immediately after co-culture with *S. aureus in vitro*.[11] This occurred without additional stimuli and as the result of both direct and indirect interactions. Under these circumstances, EETs were found to entrap and inhibit the growth of *S. aureus*.[11] The observation that extracellular nucleases are found in various pathogenic bacteria including *S. aureus,* support the hypothesis of a pathophysiological relevance for extracellular DNA traps.[14]

In recent years, EETs have been linked to various infectious and noninfectious diseases, including inflammatory skin and intestinal diseases. Unsurprisingly, EET formation is often observed in allergic diseases, such as bronchial asthma, contact dermatitis, atopic dermatitis (AD) and allergic drug reactions.[9,15-17] In eosinophilic esophagitis and bullous pemphigoid, an increased presence of EETs is observed at sites of epithelial barrier defects.[17] Therefore, EET formation could be an important mechanism for protecting against infections, but might simultaneously cause damage to the surrounding tissue, further compromising the epithelial barrier.[12] As epithelial barrier defects are more and more recognized as a key factor in various diseases, it is likely that EET formation may contribute to the pathophysiology of chronic airway diseases.

Altogether, these findings make eosinophils, EETs and their possible interactions with *S. aureus* an interesting field of study in chronic airway diseases. Data from our group have shown that CRSwNP is associated with eosinophilic infiltration, increased IL-5 and eotaxin [3], but also IL-33 and TSLP levels (unpublished data) and a consistent colonization with *S. aureus*. The presence of EETs was shown in endobronchial biopsies from asthma patients and in secretions of eosinophilic chronic rhinosinusitis patients.[9,18] However, the dynamics of their activation and the factors regulating their release have not been clarified in human diseased mucosal tissue. We aimed to understand the relevant mechanism behind this activation in the context of clinical patient samples.

2. Materials and Methods

2.1. Sample collection

Nasal polyp tissue samples from 15 patients undergoing endoscopic sinus surgery for chronic rhinosinusitis with nasal polyps (CRSwNP) and inferior turbinates (IT) from healthy individuals (5 patients undergoing septal surgery because of anatomical deviations) were collected. For the isolation of peripheral blood eosinophils, whole blood was collected from healthy volunteers. Written informed consent was obtained from all patients prior to enrollment in the study. Depending on the experiment, the tissues were either used immediately, snap frozen and/or embedded in paraffin. The study was approved by the local Ethics Committee (2015/0883) and the regulatory authorities of Belgium. None of the patients received intranasal, oral and/or intramuscular corticosteroids within the 4 weeks before surgery. For female subjects, pregnancy or lactation was excluded. Patient characteristics are summarized in Table 1.

2.2. Isolation of peripheral eosinophils and migration assay.

Blood eosinophils were purified using the granulocyte fraction after whole blood Ficoll-paque centrifugation from healthy donors using the CD16 depletion kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated eosinophils was determined as > 95% by May-Grünwald-Giemsa (MGG) staining and light microscopy.

For the migration assay, eosinophils were primed for 20 minutes with 50 ng/ml IL-5 (PeproTech) and then allowed to migrate through 5 µm pore size poly (vinylpyrrolidone)-free polycarbonate filters (VWR International) for 90 minutes at 37°C. The lower compartment contained RPMI 1640 with 5% Bovine Calf Serum (Life Technologies) and was considered as control tissue culture medium (TCM). TCM containing 10 ng/ml eotaxin (PeproTech) was used as a positive control. Three days before the experiment primary epithelial cells, isolated from nasal polyp tissue as described previously [19], were

seeded (BEGM medium, Lonza) in the basolateral compartment. Before the migration assay, the medium was changed to TCM medium with or without $2x10^7$ CFU *S. aureus*.

After the migration assay, the membrane was subjected to MGG staining and its lower side was evaluated for the number of migrated eosinophils. The chemotactic index was calculated by dividing the number of cells migrating under the experimental condition by the number of cells migrating under TCM condition.

2.3. Immunofluorescent staining

The tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After rehydration of the tissue slides (5 µm), they were subjected to different staining procedures.

A. EET staining

EETs were visualized in paraffin-embedded tissue slides (5 µm) by means of indirect immunofluorescence followed by counterstaining for DNA. After blocking and incubation with the polyclonal anti-major basic protein (MBP) (Monosan) antibody (1:50), the slides were incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:400) (Life Technologies) and the DNA was stained by incubation with propidium iodide (1 µg/ml) (Sigma Aldrich). Subsequently the slides were analyzed with a confocal laser-scanning microscope (Leica MicroSystems). Since the EETs are difficult to detect in fixed tissues, staining was performed on three different tissues for each patient. For each patient and each piece of tissue, 5 fields were selected in the studied regions (stroma, subepithelial or at epithelial defects). In those fields, the amount of EETs was counted and normalized for the amount eosinophils. EETs were expressed as % of eosinophils generating EETs throughout the manuscript.

B. Immunohistochemistry staining

After blocking, the tissue slides were incubated with the primary antibody. Primary antibodies for MBP (Monosan) and Caspase-3 (1:200) (Cell Signaling Technology) were used for the staining of

eosinophils and apoptotic cells respectively. The immunohistochemistry stain was further performed using a REAL Detection System, Alkaline Phosphatase/RED kit (Dako) according to the manufacturer's instructions.

2.4. *S. aureus* staining

Paraffin sections of nasal polyp tissue (for CRSwNP patients) or IT (for normal control patients) were rehydrated and incubated for 30 minutes with the *S. aureus* probe using PAN-fish kit (AdvanDx Company) at 55°C. After incubation, the unbound residual probe was removed by incubation with wash solution (AdvanDx Company) for 30 minutes at 55°C. After washing, the slides were counterstained with propidium iodide (1 μg/ml) (Sigma Aldrich) for 10 minutes at room temperature. Finally the slides were mounted and examined using a fluorescence microscope. Patients and controls were screened in triplicate for *S. aureus* and received a score as follows: '0' no colonization; '1' planktonic colonization, not more than two bacteria in close proximity; '2' planktonic colonization, more than two bacteria in close proximity; '3' colonization with biofilm formation.

To stain both *S. aureus* and EETs, the procedure was performed as described in 2.3. The slides were washed with PBS and subjected to the immunofluorescent staining as described above with following changes: a red fluorescent protein -conjugated secondary antibody (1:400) (Life Technologies) was used and the DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies).

2.5. *Staphylococcus* exposure experiments

S. aureus (kind gift of Prof. Dr. von Eiff, Münster, Germany) and *S. epidermidis* (ATCC 14990) were grown overnight in tris-buffered saline (TSB) and diluted in RPMI:DMEM (Dulbecco's Modified Eagle medium) (1:1) (Life Technologies) to $2x10^7$ CFU/ml. Patient tissues were kept submerged in 2 ml of the bacteria solution or in tissue culture medium and maintained for 2 hours at 37°C. Tissues submerged in RPMI:DMEM medium, tissue control medium (TCM), was considered as control tissue. Subsequently, the tissues were placed on a stainless steel grid and incubated at air-liquid interface

for 30 minutes, 1 and 2 hours. For the inhibition experiment, the tissue cubes were pre-incubated (submerged) with 10 µM of the NADPH oxidase inhibitor diphenyleneiodionium chloride (DPI) in RPMI:DMEM for 1 hour at 37°C before proceeding as described above. After incubation, the tissues were immediately fixed in 4 % paraformaldehyde and/or snap frozen in liquid nitrogen.

2.6. Reactive oxygen species measurements

The tissues were immediately fixed in 4% paraformaldehyde and embedded in paraffin. Upon rehydration, tissue slides were incubated with 5 µM dihydroethidium (Sigma Aldrich) for 30 minutes. After washing steps with PBS, the slides were mounted with Vectashield anti-fade mounting medium (Vector Laboratories). The slides were evaluated under a confocal fluorescent microscope and pictures were taken while keeping the parameters (12 µs exposure time, 13% laser intensity) constant. For each experiment (n=3), 10 fields in the subeptihelial region (< 100 µm distance from epithelium) per condition were selected randomly, and assessed/measured by 2 (blinded) individuals. The fluorescent mean intensity was quantified and normalized for surface area by measuring the integrated fluorescent density using ImageJ analysis software.

2.7. Immunoblot analysis for TSLP

Protein lysates from patient tissues were made in RIPA buffer containing protease inhibitors (Sigma) as described previously.[20] The concentration of protein in the lysates was determined using a protein assay (Bio-Rad) with bovine serum albumin as a protein standard. The lysates were mixed 1:1 tricine sample buffer (Bio-Rad) with 2% β-mercaptoethanol. Thirty micrograms of protein lysate was loaded on a tris-tricine 16.5% precast gel (Bio-rad). Proteins were transferred to nitrocellulose membrane and incubated in 5% bovine serum albumin overnight. Long form TSLP (lfTSLP) and short form TSLP (sfTSLP) protein were detected using anti-TSLP antibody (1:1000)(Abcam). The secondary antibody used was anti-rabbit Ig donkey whole Ab-HRP linked (1:6000) (NA934, GEAkta). Reactive bands were visualized using SuperSignal West Femto maximum sensitivity substrate (ThermoFisher Scientific).

2.8. Real-Time PCR

Snap frozen tissue samples (± 30 mg) were disrupted and thawed directly into lysis solution (QIAGEN) followed by homogenization using a QIAshredder homogenizer (QIAGEN). Subsequently, RNA was isolated using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. RNA quality was assessed with an Experion Automated Electrophoresis System (Bio-Rad Laboratories, Belgium) and cDNA was synthesized from 1 µg of RNA using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad). Quantitative real-time PCR was used to quantify mRNA levels of TSLP. Amplification reactions were performed on a Light Cycler LC480 System (Roche) by using a specific PrimePCR Assay (Bio-Rad). qPCR reactions contain 5 ng cDNA (total RNA equivalent), 0.25 µl PrimePCR primermix (Bio-Rad), 2.5 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a final volume of 5 µl. PCR protocol consisted of 1 cycle at 95°C for 2 minutes followed by 44 cycles at 95°C for 5 seconds, at 60°C for 30 seconds and at 72°C for 1 second, and a dissociation curve analysis from 60°C to 95°C. After a validation with geNorm (Biogazelle, Belgium), two reference genes, elongation factor 1 (EF-1) and succinate dehydrogenase complex flavoprotein subunit A (SDHA), were used to normalize for transcription and amplification variations among samples. Primer sequences are shown in Table S2. The normalized relative quantities (NRQs) were calculated with the qBase+ software (Biogazelle, Belgium) and the final gene expression results are expressed as the logarithm of NRQs per 5 ng cDNA.

2.9. Cytokine measurements

The frozen tissues were weighed, homogenized and centrifuged as described previously.[20] The samples were then assayed for IL-5, TSLP and periostin using commercially available ELISA kits from R&D Systems (Minneapolis, Minnesota, USA) and for ECP and IgE using the UniCAP method (Thermo Fisher Scientific) with the appropriate dilution factor and according to kit instructions. The samples were measured following the instructions of the manufacturer, on a Bio-Plex 200 Array Reader (Bio-Rad, Hercules, CA, USA).

2.10. Statistical analysis

Statistical analysis was performed using the SPSS version 23 software program. For between-group comparisons (Fig. 3A-C, Fig. 5C, Fig. 7AB, Fig. S1 A-D, Fig. S3 A-B, Fig. S4 A-D) the Mann-Withney U test was used. For the comparison of related samples (comparison of EET formation related to tissue localization, Fig. 4 E, Fig. 6 A-C, H), data were analyzed with a Friedman test, followed with Dunns post test. Linear correlation analysis (Fig. S3 C-D) was performed with a Spearman correlation test. Significances were expressed as *p<0.05,**p<0.01, ***p<0.001, p-values less than or equal to 0.05 were considered as statistically significant.

3. Results

Subepithelial eosinophils in nasal mucosa of patients with CRSwNP

CRSwNP patients selected for the study (Table 1) had significantly elevated tissue levels of IgE, IL-5 and ECP (Fig. S1 A-C) compared to the normal controls. Significant number of eosinophils (Fig. S1D and Fig. S1 E-F, $p < 0.001$), and to a much lesser extent neutrophils (Fig. S1D, $p < 0.05$) were elevated in the tissues of the diseased population.

No infiltration or alignment of eosinophils was observed in the IT control population (n=5, Table S1 and Fig. 1A). In the tissue of the control population, eosinophils were rarely observed, even in the allergic individuals. An increased presence and alignment of eosinophils underneath both intact (Fig. 1B) and barrier defective epithelium (Fig. 1D-E) was observed in all CRSwNP patients Table S1, n=15). In some patients however, the alignment was more pronounced than in others. Regions with completely denuded epithelium (Fig. 1E) were always associated with subepithelial infiltrates of eosinophils. In the latter case, the subepithelial region could range from large and diffuse depositions of MBP or free granules with very few to no intact eosinophils showing clear tissue damage (Fig. 1E), to infiltrations of large numbers of intact eosinophils, depending on the patient and the localization within the polyp. Regions with visually deviant epithelium (e.g. single cell layers, missing epithelial cells) were often associated with larger and denser zones of subepithelial eosinophils Fig. 1C).

Increased presence of EETs in subepithelial regions

Although there were substantial differences between patients, we observed EETs in all of the 15 patients analyzed. Considering all patients, the mean fraction of EET-releasing eosinophils in the subepithelial region (selected as region < 100 µm distance from epithelium) was 8.8 ± 4.8 % of the infiltrating eosinophils, but ranged from < 2 % to 15 % of the eosinophils contributing to EET formation (Fig. 2A). The proportion of EET-releasing eosinophils was differently distributed throughout the polyp. Deep in the stroma, eosinophils were found mainly intact (Fig. 2B) (and

sometimes degranulated) and EET formation was very rare (0.2 ± 1.1% of the eosinophils); subepithelially (< 100 µm distance from epithelium) denser eosinophilic infiltrates (Fig. 2C) were located wherein eosinophils were found intact, or degranulating, releasing single EETs (Fig. 2D) and/or clusters of eosinophils releasing EETs (Fig. 2E). The long and thin EET structures often seemed to align underneath the epithelium, and sometimes even seemed to connect the EET releasing eosinophils with other cells including other eosinophils. In most of the specimens we observed more clusters of eosinophils releasing EETs than single EET releasing cells. No EET- releasing eosinophils were found in the control ITs. No correlation was found between the extent of EET formation and presence of atopy or asthma.

Subepithelial regions with enhanced EET formation are not correlated with the presence of apoptotic epithelial cells.

In the subepithelial region (< 100 µm distance from epithelium), the proportion of eosinophils generating EETs was in general higher than in the stroma. The subepithelial regions with visual deviant epithelium showed a significant increase of EETs (3.1 ± 1.9 fold, $p < 0.01$), compared to subepithelial regions with intact epithelium. These abnormal epithelial regions with increased subepithelial eosinophilic infiltrates (Fig. S2 A) were not characterized by apoptotic (epithelial) cells (Fig. S2 B), as indicated by an IHC stain for caspase-3. We also observed no abnormal nuclear morphology in infiltrating eosinophils, indicating that cell lysis did not occur under the conditions of EET formation (Fig. S2 B, inset B').

EET formation is associated with elevated IL-5 and periostin levels, and with *S. aureus* infection

CRSwNP patients with higher numbers of infiltrating eosinophils had significantly higher (relative) amounts of EET formation ($p < 0.05$, Fig. S3 A). Patients with EETs were characterized by significantly higher IL-5 levels in the tissue (Fig. 3A). Periostin levels were significantly increased in CRSwNP patients, compared to controls ($p < 0.01$, Fig. S3 B) and were positively correlated with IL-5 levels ($p < 0.01$, $R^2=0.665$, Fig. S3 C). In addition, patients with more EETs had significantly increased periostin

levels in the tissue (Fig. 3B). Interestingly, no correlation between EET formation and TSLP protein concentration (Fig. S3 D) was found in the tissue.

Because of the well-known association between *S. aureus* colonization and IL-5, all patients (n=15) were evaluated and received a score (Table 2) for the *S. aureus* infection status as indicated in the Materials and Methods section. The group of CRSwNP patients with the highest percentages (10% < x < 20%, Fig. 3C) of eosinophils generating EETs, had a significant higher score for *S. aureus* colonization than the patients with the lowest (< 5%, Fig. 3C, p≤ 0.05) and intermediate EET levels (< 10 %, Fig. 3C, p≤ 0.01).

Based on findings described above, five patients with high EET formation and *S. aureus* colonization were selected and screened for co-localization of *S. aureus* and EETs in the tissue. In 3 of the 5 patients, *S. aureus* was found entrapped in an EET in the tissue (Fig. 3D). Other EETs were often observed in the neighborhood of *S. aureus* bacteria.

In vitro exposure of _S. aureus,_ but not _S. epidermidis,_ leads to increased EET formation in tissue fragments

After one hour exposure of CRSwNP tissue fragments to *S. aureus* in air-liquid culture, EET formation was markedly enhanced (3.5 ± 1.2 fold increase, Fig. 4B and Fig. 4E) with up to 60% of eosinophils (local effect) contributing to EETs as compared to the TCM control (Fig. 4A). The traps formed were connected with each other as well as with other cells. After 2 hours exposure in air-liquid culture, we noticed the increased presence of eosinophils releasing EETs at sites where *S. aureus* was observed (4.2 ± 0.9 fold increase, Fig. 4C and Fig. 4E). The EET formation was concentrated in the subepithelial regions (Fig. 4C) and, more specifically, at sites with epithelial defects and near *S. aureus*. These findings were in strong contrast to the results of similar exposure experiments with *S. epidermidis* (Fig. 4D) where no increased EET formation (Fig. 4E) could be observed at any time point.

Eosinophils migrate towards and entrap *S. aureus* in an *ex vivo* diseased human mucosal tissue model

In the *ex vivo* human tissue model, after 2 hours air-liquid exposure, large numbers of eosinophils were concentrated (compared to the general distribution of eosinophils in the tissue) at the sites of epithelial defects, having sometimes even migrated out of the tissue. The eosinophils trapped *S. aureus* at sites of epithelial defects (Fig. 5A), but also at sites with an intact, but single cell-layered epithelium (Fig. 5B). Active migration of eosinophils towards *S. aureus* was confirmed by an *in vitro* migration assay (Fig. 5C). In addition, the migration towards *S. aureus* was enhanced by the combined presence of *S. aureus* and epithelial cells and had a tendency to further increase further with longer co-incubation times (4 hours).

Isolated cells from nasal secretions of CRSwNP patients were shown to be extremely reactive with massive EET formation upon *ex vivo* challenge with *S. aureus* for 15 minutes without the need for priming. The bacteria were found entrapped in the massive EET network (Fig. 5D).

***In vitro* generation of EETs in response to *S. aureus* is associated with elevated production of reactive oxygen species**

Measurements of reactive oxygen species (ROS) in the tissue revealed a significant increase in ROS production after exposure to *S. aureus*. The production of ROS was significantly enhanced after 30 minutes (Fig. 6A) and one hour exposure to *S. aureus* as compared either to the TCM control or to a comparable exposure to *S. epidermidis* (Fig. 6B). After 2 hours, no further significant differences in ROS production between the different *in vitro* conditions were observed (Fig. 6C). The role of ROS and NADPH oxidase was confirmed by pretreatment of the tissue with DPI (Fig. 6D-G and Fig. 6H) which was able to block totally (Fig. 6G and Fig. 6H) the extensive EET formation caused by exposure to *S. aureus* (Fig. 6E).

Periostin and TSLP levels were not significantly increased after *in vitro* exposure to *S. aureus*

Periostin (Fig. 7A) and TSLP levels (Fig. 7B) were not significantly elevated in tissue homogenates after exposure to *S. aureus*, as determined by ELISA. Immunoblot analysis (Fig. 7C) showed that the short form TSLP (sfTSLP) was undetectable, while the expression of long form TSLP (lfTSLP) was not significantly altered between the different conditions. At the mRNA level, lfTSLP was elevated after 2 hours exposure to both *S. aureus* and *S. epidermidis* (Fig. 7D), although in the latter case the increase was not statistically significant. ELISA measurements of supernatant showed only detectable (secreted) TSLP levels after 2 hours exposure to *S. aureus* (data not shown), but in none of the earlier time points was TSLP detectable in the supernatants.

4. Discussion

The generation of EETs has been linked to various pathologies, including inflammatory skin and intestinal diseases. Unsurprisingly, EET formation was also observed in allergic diseases, such as bronchial asthma, contact dermatitis, atopic dermatitis (AD) and allergic drug reactions.[9,15-17] Our aim was to investigate the presence of EETs in CRSwNP tissues. Due to the eosinophilic environment and the increased levels of IL-5, eotaxin and TSLP, we believed this was an ideal context for the generation of EETs. Our data have now shown that EETs are also formed in the nasal polyp tissues. An increased presence of eosinophils and EETs was observed at subepithelial sites, especially at sites with epithelial defects. These data are in line with similar observations described in eosinophilic esophagitis and bullous pemphigoid.[21] Our research in patient samples showed that about 9% of the eosinophils present contribute to EET formation. It is very likely that EET formation *in vivo* is much more frequent as EETs are generated in a very short time frame, and possibly rapidly degraded.

The relationship between *S. aureus* and elevated levels of IL-5 and CRSwNP has been recognized for years.[5,13] The antibacterial properties of EETs and their formation as a response to *S. aureus in vitro*, made it interesting to study this relationship in the context of clinical tissue samples from CRSwNP patients. Our data have now shown that EET formation is associated with increased IL-5 levels, but

also with *S. aureus* colonization in patient's tissues. In addition, we show for the first time direct proof of *S. aureus* entrapment by EETs in patient's tissues. Furthermore, we were able to show the induction of EET formation after exposure to *S. aureus*, but not to *S. epidermidis* in tissues of CRSwNP patients. The presence of DNA traps in secretions of eosinophilic chronic rhinosinusitis patients and their role in increasing the viscosity of these secretions has already been reported.[18] Our experiments now demonstrate that eosinophils migrate towards *S. aureus* and entrap the organism both inside and outside the human diseased mucosal tissue. Eosinophils from CRSwNP patient tissues showed a massive reactivity towards *S. aureus* exposure with extensive EET formation. This suggests that the reaction in the patient and at the diseased location could be far more extensive than currently is recognized from *in vitro* exposure experiments using peripheral eosinophils. In contrast to the situation in diseased tissue, *in vitro* exposure also needed prior priming for EET formation. These data also imply that a direct contact between the bacteria and the eosinophil is likely to initially trigger EET formation. This could also explain the increased EET formation at sites of epithelial defects.

Our results imply that eosinophils are likely to be specifically recruited to and generate EETs at sites of epithelial damage, possibly to protect the host from infections. In nasal polyps, several defects in the mucosal defense have been shown, including increased levels of M2 macrophages, a decrease in β-defensin 2 and 3, defective epithelial tight junctions, a compromised expression of Toll-like receptor 9, etc.[22-25] In this situation, eosinophil-mediated immune responses, like EET formation, might be crucial for maintaining the barrier function after inflammation-associated epithelial cell damage, protecting the host from an uncontrolled invasion of bacteria.

This hypothesis is further supported by, (i) the increased presence and alignment of eosinophils at subepithelial sites, (ii) the observed migration and entrapment of *S. aureus* in the *in vitro* exposure model and (iii) the *in vitro* migration assay confirming an active migration of eosinophils. As the cytotoxicity of eosinophilic granule proteins is well known, our observations could also point to a more prominent role of eosinophils in damaging or preventing the repair of the epithelial barrier in

CRSwNP.[21,26,27] Whether the observed EETs are a causal effect of damage to the epithelial barrier, or (partly) the source of epithelial damage, will be the subject of future investigations.

Recently the subepithelial deposition and association between periostin and eosinophilic inflammation was shown in CRSwNP.[28] This made periostin an interesting candidate to investigate in association to EET formation. Indeed, we found significant elevated periostin levels in patients with more EETs. Despite this fact, no increase in periostin was found after experimental exposure to *S. aureus*. This can possibly be attributed to the short time frame wherein we studied the trap formation. In addition, periostin could facilitate the infiltration of eosinophils in the tissue, as reported for allergic lungs and eosinophilic esophagitis.[29] Therefore, periostin could be increased as a consequence of the Th2 context without being directly involved in EET formation.

Interestingly, the induced generation of the EETs was observed within a narrow time frame (1 hour). With these observations, questions arise about the trigger of EETs release in the tissue after exposure. Our experiments have clearly shown that, upon stimulation with *S. aureus*, ROS levels are increased within the same time frame. Previously, it had been demonstrated that inhibition of NADPH oxidase activity, responsible for ROS production, also inhibited EET formation by eosinophils *in vitro*.[10,11] Our data have shown that inhibition of NADPH oxidase was also able to block ROS production and EET formation in human mucosal tissue, implying an important role of NADPH oxidase activity in EET formation following *S. aureus* challenge *in vivo.*

To our knowledge, a similar role for eosinophilic granule proteins has not been reported. We feel that solving this question is beyond the scope of our manuscript and would imply a separate EET-based study. However, would like to thank the reviewer for highlighting this as we feel this could be a valuable hypothesis for our future research.

IL-5 and TSLP are both well-known triggers of EET formation *in vitro*.[10,11,16] Therefore IL-5 and TSLP were investigated as potential triggers for EET formation in CRSwNP tissues. Unsurprisingly, increased IL-5 levels were indeed associated with more EETs. Interestingly however, TSLP levels were

not markedly enhanced in the tissue as determined by ELISA. This was in contrast to our expectations since TSLP is known to be elevated after epithelial damage, was correlated with EET release in active eosinophilic esophagitis and had been reported to directly trigger EET formation.[11,21,31]. Previously, EETs were described as capable of entrapping and killing *S. aureus* and *S. epidermidis in vitro*. In contrast to *S. aureus*, direct contact with *S. epidermidis* was not sufficient for EET release and was dependent on the addition of TSLP to execute this effect.[11] This could explain why EETs were mainly concentrated at sites of epithelial defects (direct contact with S. aureus) and why *S. epidermidis* was unable to induce EET formation in the experimental human mucosal exposure experiments, as TSLP levels were not elevated in the time frame studied. PCR data however, showed an increase in TSLP production after two hours. Therefore, it is possible that at later time points, TSLP could still play a role in EET formation.

Altogether these data imply that the tissue context, the nature of the trigger and the interplay between these two, play an important role in determining the intensity of EET formation. It is likely that the initiation of EET formation is triggered by direct contact between *S. aureus* and eosinophils and that, as a consequence of secondary signaling events, EET formation in later stadia is further supported by increased IL-5, TSLP or other triggers at later time points.

In neutrophils it was shown that, besides ROS, also the granule proteins neutrophil elastase and myeloperoxidase had a regulatory role in the formation of NETs. [30] Eosinophilic granule proteins could possibly exert a similar function in EET formation but this is subject of future research.

Although EET formation seems to have a beneficial role by targeting S. aureus, the targeting of eosinophils in eosinophils-associated diseases is highly effective. This paradox raises questions about cause and consequences in the pathophysiologic role of epithelial barrier defects, germs such as S. aureus, and the type-2 inflammatory reaction.

In conclusion, we describe here for the first time the formation of EETs in nasal polyp tissue; their localization, dynamics and relationship with the presence of *S. aureus*, suggesting a role for EETs in

epithelial barrier defects. ROS could be identified as essential requirement for EET formation by eosinophils. These findings yield new insights into the possible role of EETs in human airways, and link *S. aureus* to eosinophilic airway disease such as CRSwNP and co-mordid asthma.

5. Acknowledgements

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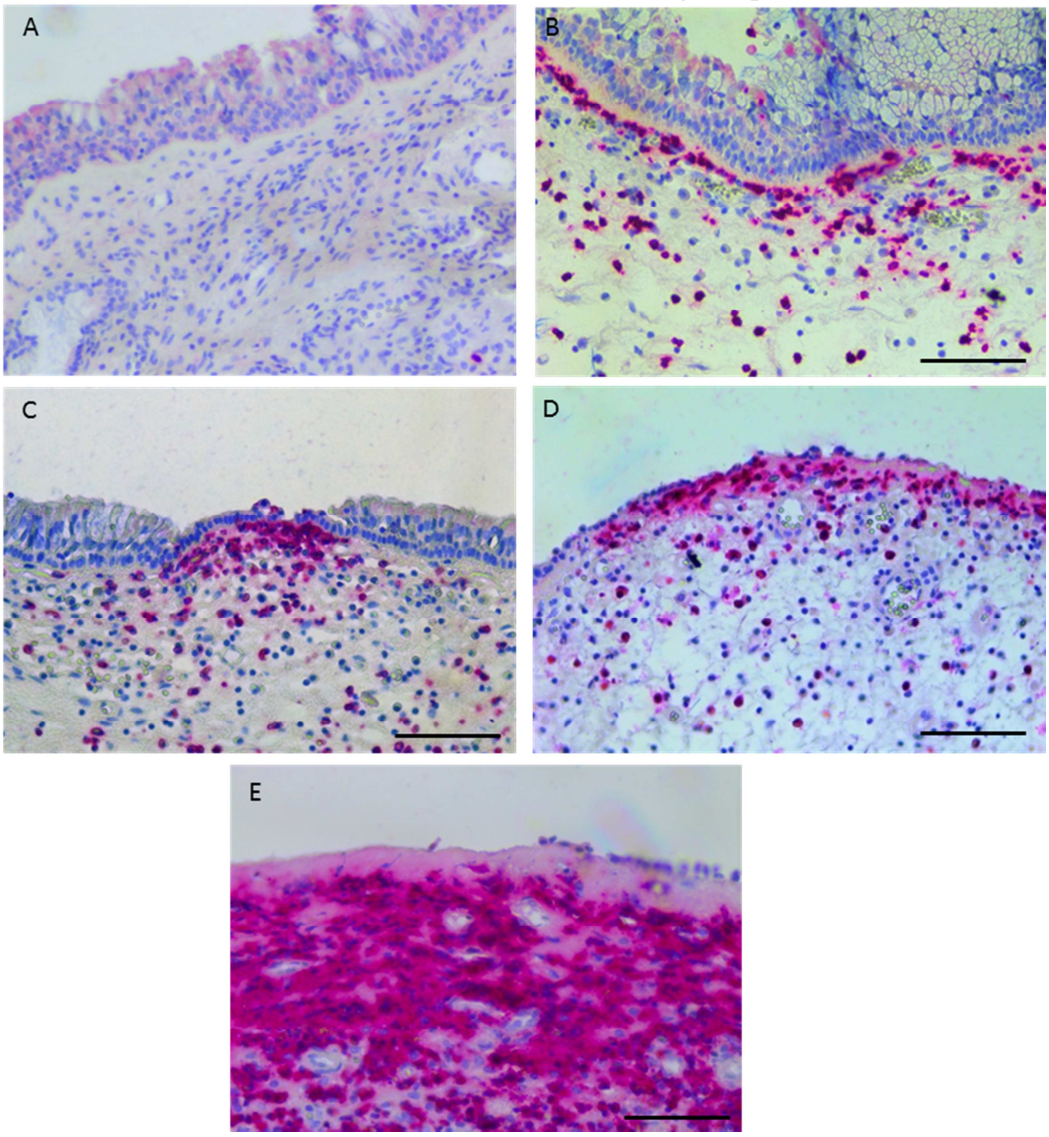
6. Figures

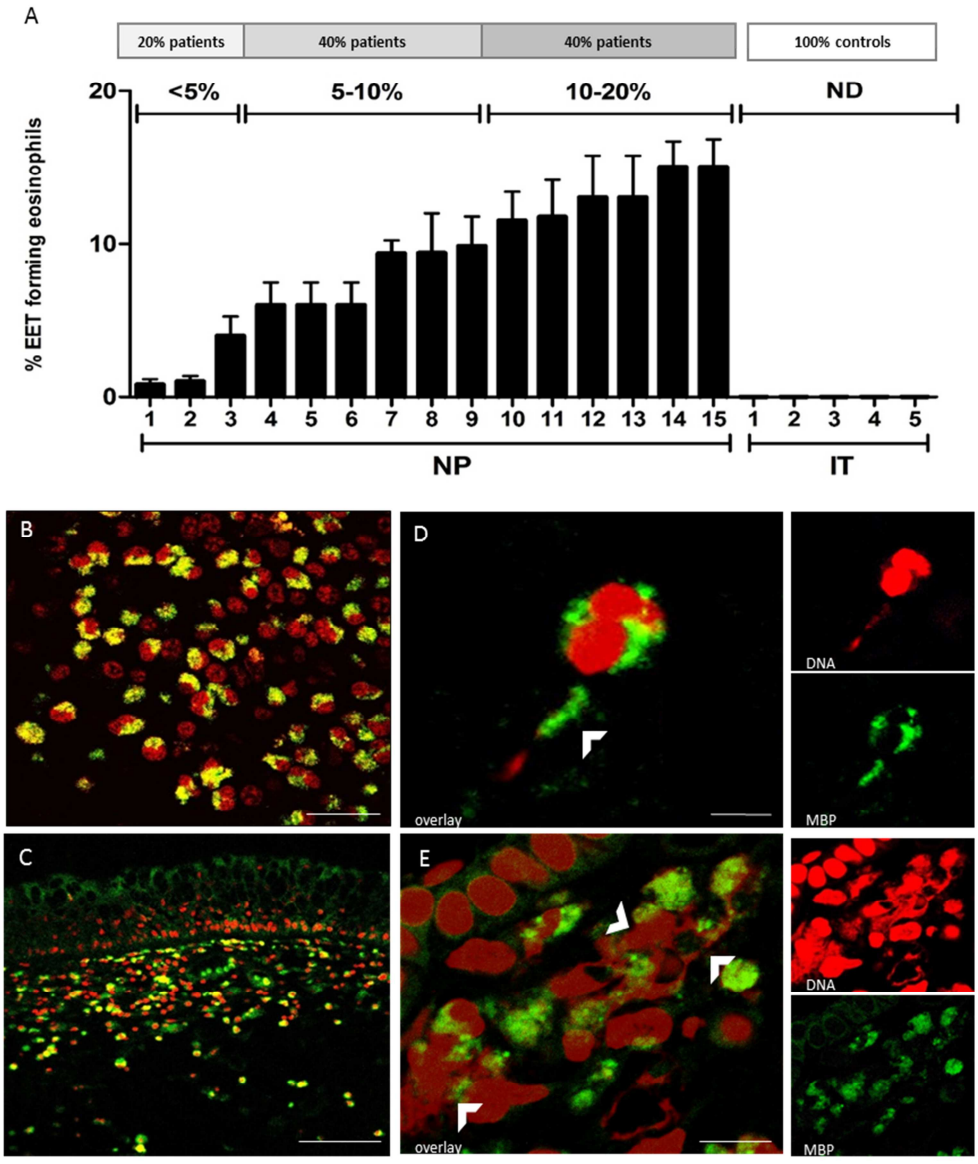
		CRSwNP	Controls
Total cases		15	5
Gender	M/F	10/5	3/2
Age	Median (range)	35 (21-92)	33 (29-42)
Ethnicity		Caucasian	Caucasian
Allergy	-/+	2/12	2/2
	missing cases	n=1	n=1
Asthma	-/+	7/7	1/4
	missing cases	n=1	n=0
Treatment asthma	-/+	4/3	2/2
IgE	Mean ± SD	1448.64 ± 1935.70	62.03 ± 77.95
SAE-IgE	Mean ± SD	6.85 ± 7.41	BDL

Table 1. Patient characterization. Chronic rhinosinusitis with nasal polyps (CRSwNP), Male (M), Female (F), present (+), not present (-), below detection limit (BDL), standard deviation (SD), immunoglobulin E (IgE), *S. aureus* specific immunoglobulin E (SAE-IgE). Allergy was defined as 'present' when the patient has a positive skin prick test for at least one of the allergens commonly tested in our region.



549

550 **Fig. 1 Eosinophils in nasal mucosa tissue.** (A-E) Immunohistochemical stain for MBP (red) in the
551 subepithelial region of inferior turbinate from a control patient (A) and polyp tissue from different
552 patients (B-E) showing the localization of eosinophils and different intensities of subepithelial
553 localization. Nuclei (blue) are counterstained with hematoxylin. (Scale bar = 100 µm)



554

555 **Fig. 2 Eosinophils and their DNA traps in nasal polyp tissue.** (A) Quantification of EETs in the
556 subepithelial region of patients (NP 1-15) and control (IT 1-5) tissues, EETs were expressed as % of
557 eosinophils generating EETs relative to the total amount of present eosinophils. (B-E)

Immunofluorescent staining of MBP (green) and DNA (red). (B) Intact eosinophils in the stroma (scale bar = 25 µm), (C) increased subepithelial eosinophilic aggregation (scale bar = 50 µm), (D) a single eosinophil is shown generating an EET (indicated by an arrowhead) (scale bar = 5 µm) (E) a cluster of eosinophils generating EETs (indicated by arrowheads). (Scale bar = 10 µm).

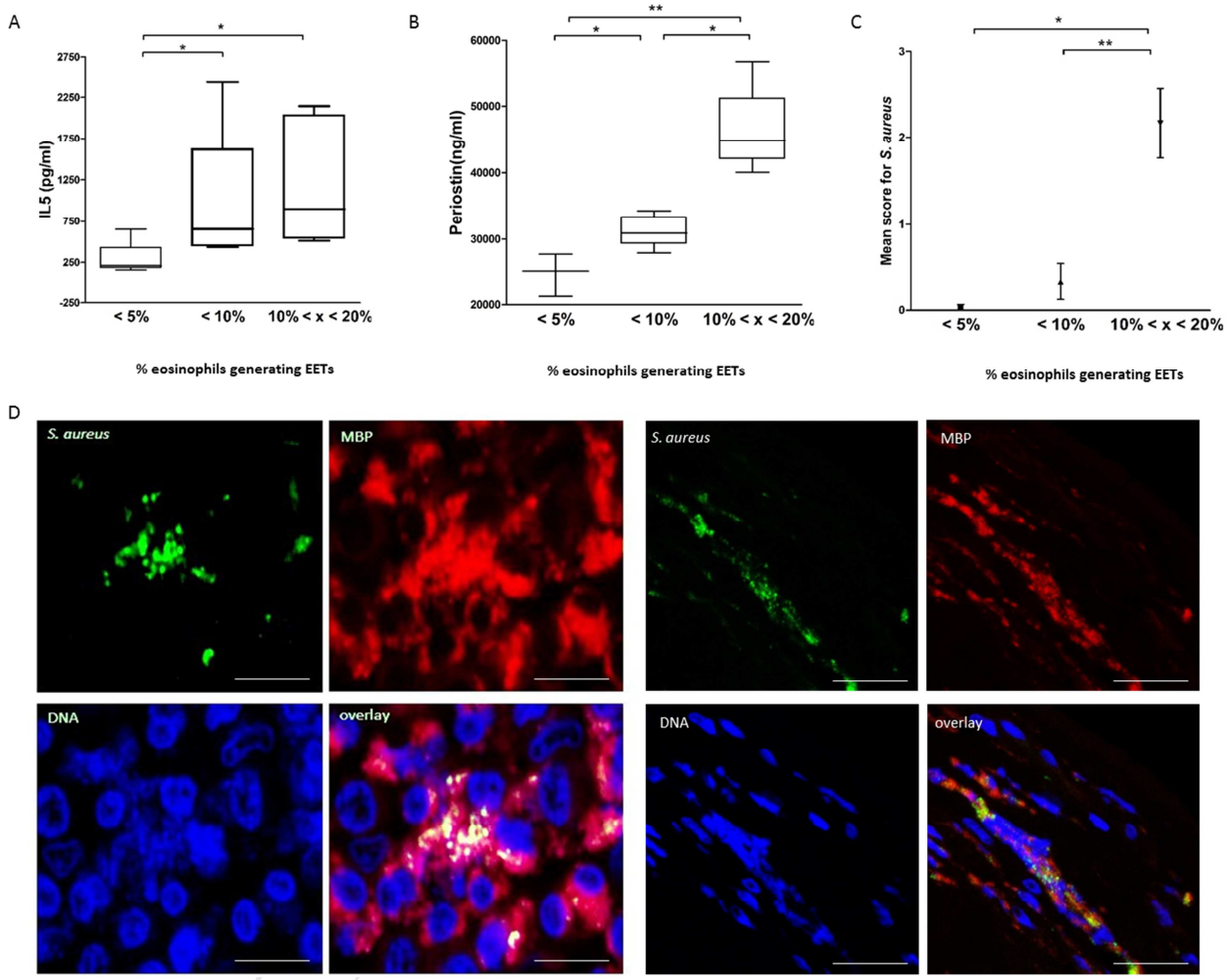
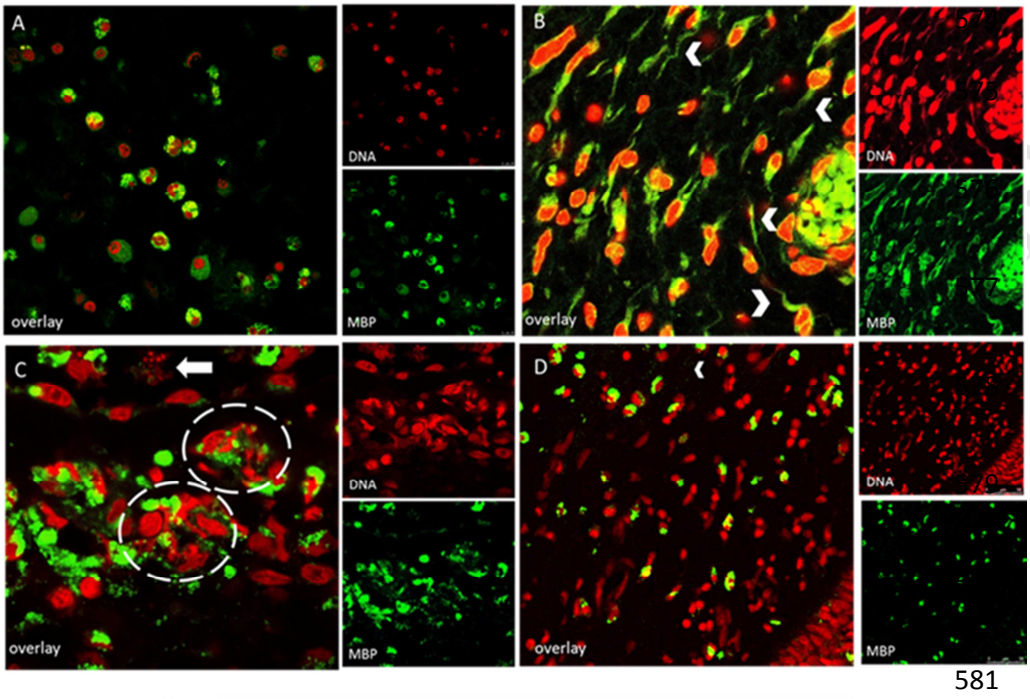


Fig. 3 Relevant percentage of eosinophils forming EETs and IL-5, periostin and *S. aureus* colonization. Tissue levels of IL-5 and periostin were measured (CRSwNP patients, n=15) by means of ELISA. *S. aureus* colonization was assessed for all patients using a PNA-Fish technique. These data were studied for bacteria association with EETs. (A) IL-5 levels were elevated in patients where higher fractions of eosinophils generating EETs were observed. (B) Periostin levels where increased where higher fractions of eosinophils generating EETs were detected. (C) In patients, increased EET formation significantly correlated with higher scores for *S. aureus* colonization. Data were analyzed by a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001. (D) Co-

571 localization of *S. aureus* and EETs. Immunofluorescent staining for MBP (red) and DNA (blue)
572 combined with a PNA-Fish stain for *S. aureus (Green).* (left picture scale bar = 25 µm, right picture
573 scale bar = 50 µm)



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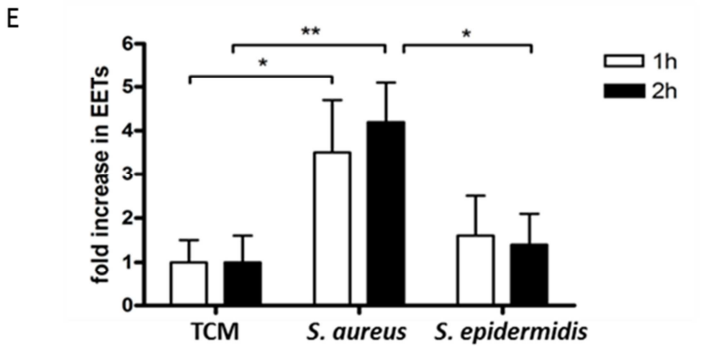


585

586 **Fig. 4 EETs in nasal polyp tissue after *in vitro* exposure to *S. aureus* or *S. epidermidis*.**
587 Immunofluorescent stain for MBP (green) and DNA (red). (A) Intact eosinophils after 2 hours air-
588 liquid exposure to tissue culture medium (control) (Scale bar = 25 µm); (B) increased EET formation
589 (arrowheads indicate some, but not all, of the EETs) after 1 hour air-liquid exposure to *S. aureus*
590 (Scale bar = 25 µm); (C) increased subepithelial formation of EETs (dashed line) in the proximity of *S.*
591 *aureus* (arrow) (scale bar = 25 µm); (D) mainly intact eosinophils after 2 hours air-liquid exposure to
592 *S. epidermidis*. (Scale bar = 50 µm); (E) Increase of EET formation in the subepithelial region after

exposure to *S. aureus* (Sa) and *S. epidermidis* (Se) compared to tissue culture medium (TCM) in nasal polyp tissue (n=3). Data were analyzed with a Friedman test, followed with Dunns post test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001

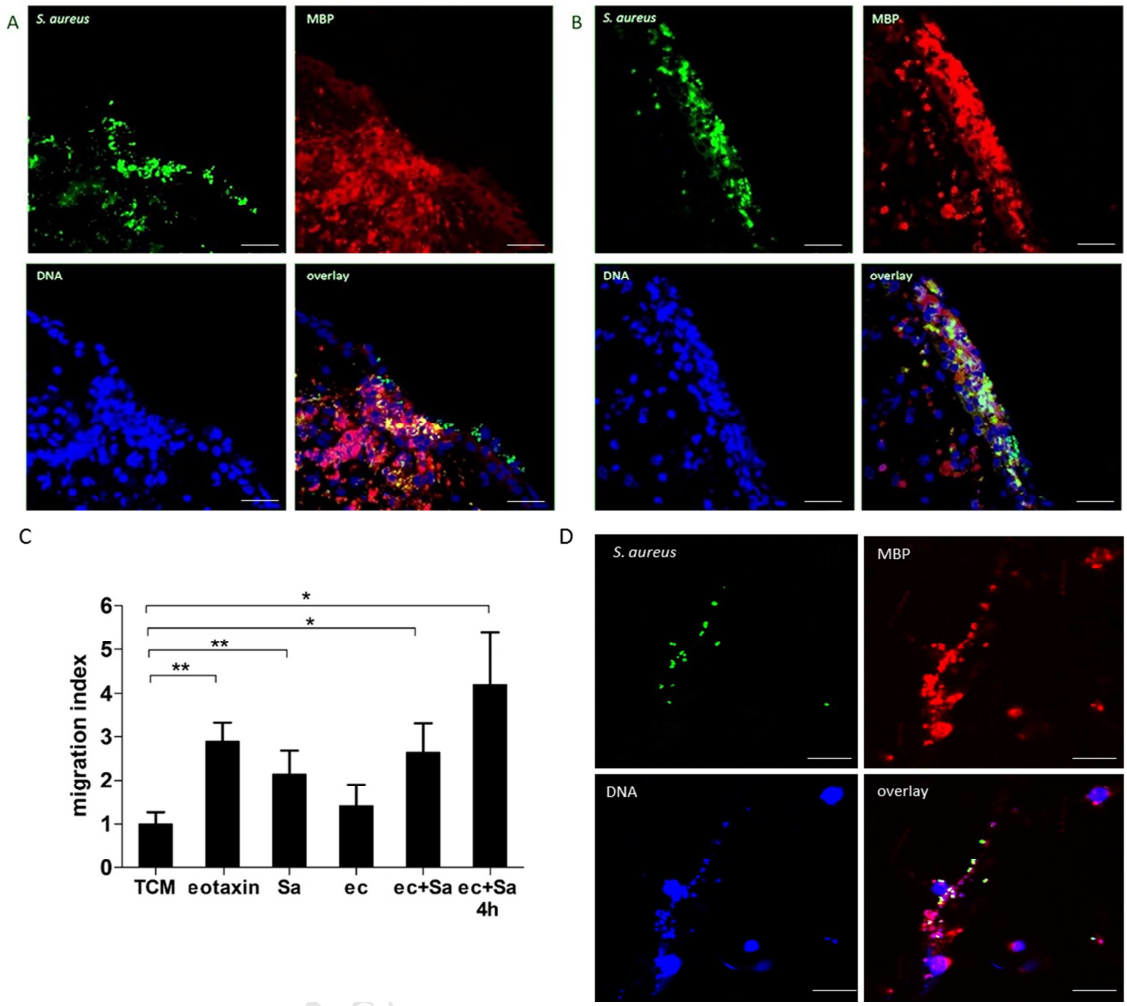
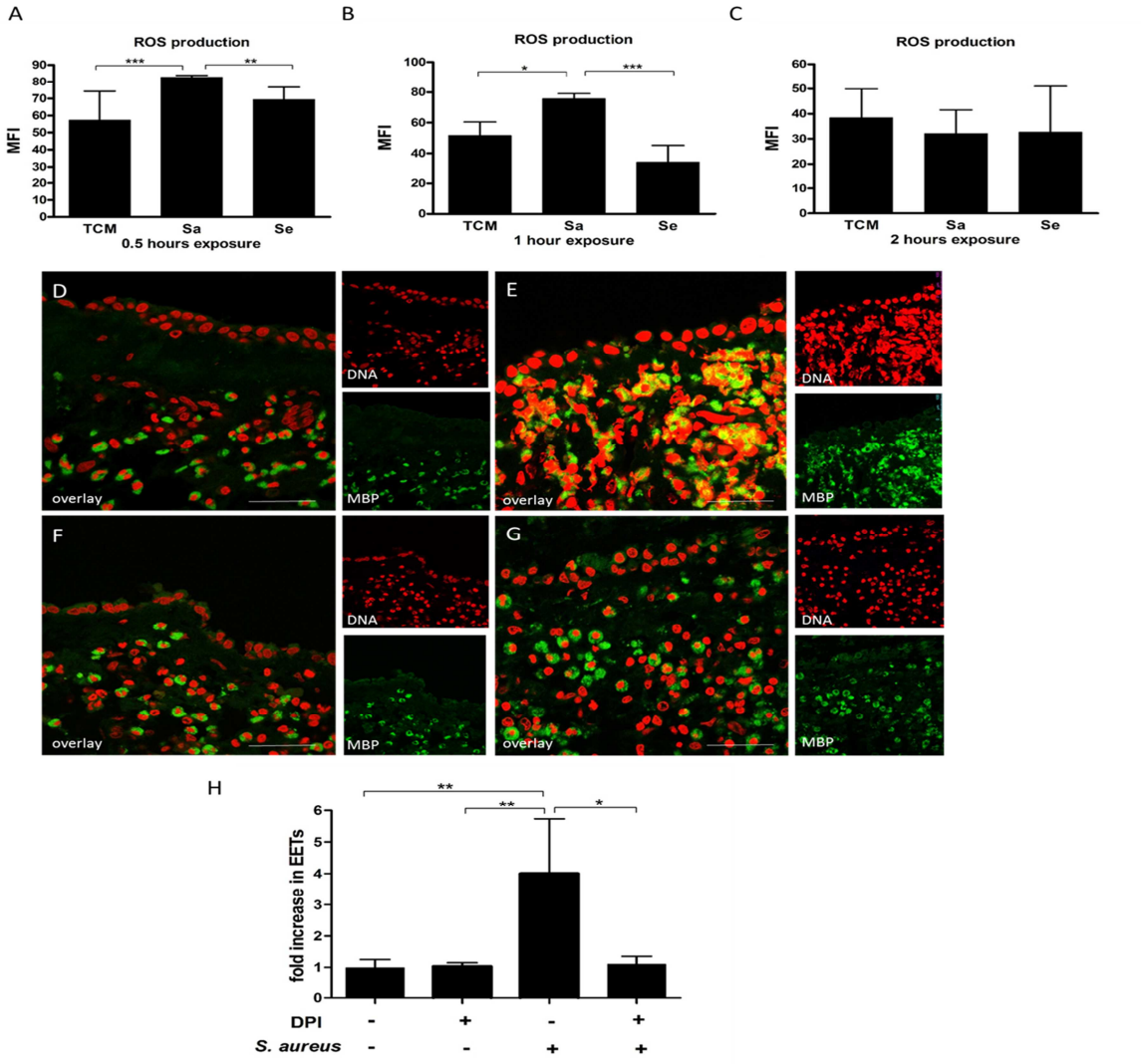


Fig. 5 Transepithelial migration and massive EET formation of eosinophils to entrap *S. aureus*. Immunofluorescent staining of MBP (red) and DNA (blue), combined with PNA-Fish stain for *S. aureus* (green) in tissue sections after 2 hours exposure to *S. aureus*. (A) Indicates the migration of eosinophils and EETs at sites of epithelial defects where direct contact with *S. aureus* is observed. (Scale bar = 25 µm) (B) Indicates transepithelial migration of a large fraction of eosinophils and the trapping of *S. aureus* in a large EET. (Scale bar = 25 µm) (C) *In vitro* migration assay of eosinophils in response to different stimuli: tissue culture medium (TCM), eotaxin, *S. aureus* (Sa), epithelial cells (ec), epithelial cells with *S. aureus* (ec+Sa), and epithelial cells with *S. aureus* after 4 hours of co-

605 incubation (ec+Sa 4h). Data were analyzed with a Mann-Whitney U test, n=3, significances are
606 expressed as *p<0.05,**p<0.01, ***p<0.001. (D) Eosinophils from a CRSwNP patient showing EET
607 formation after challenge with *S. aureus in vitro*. (Scale bar = 25 µm)



608

609 **Fig. 6 Reactive oxygen species formation after exposure to *S. aureus*, and inhibition of EET**
610 **formation by DPI.** (A-C) Production of ROS in the tissues after 0.5, 1 and 2 hours *in vitro* exposure to
611 *S. aureus* (Sa) or *S. epidermidis* (Se) (n=3). (D-G) Immunofluorescent stain for MBP (green) and DNA
612 (red). (D) intact eosinophils after 1 hours air-liquid exposure to tissue culture medium (control); (E)
613 increased EET formation after 1 hour air-liquid exposure to *S. aureus*; (F) intact eosinophils after 1
614 hour DPI treatment and 1 h air liquid exposure to tissue culture medium (control); (G) mainly intact
615 eosinophils after 1 hour DPI treatment and 1 hour air-liquid exposure to *S. aureus*; (scale bar = 100

µm) (H) Increase of EET formation after exposure to *S. aureus* and inhibition by DPI treatment in nasal polyp tissue (n=3). Data were analyzed with a Friedman test, followed with Dunns post test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001

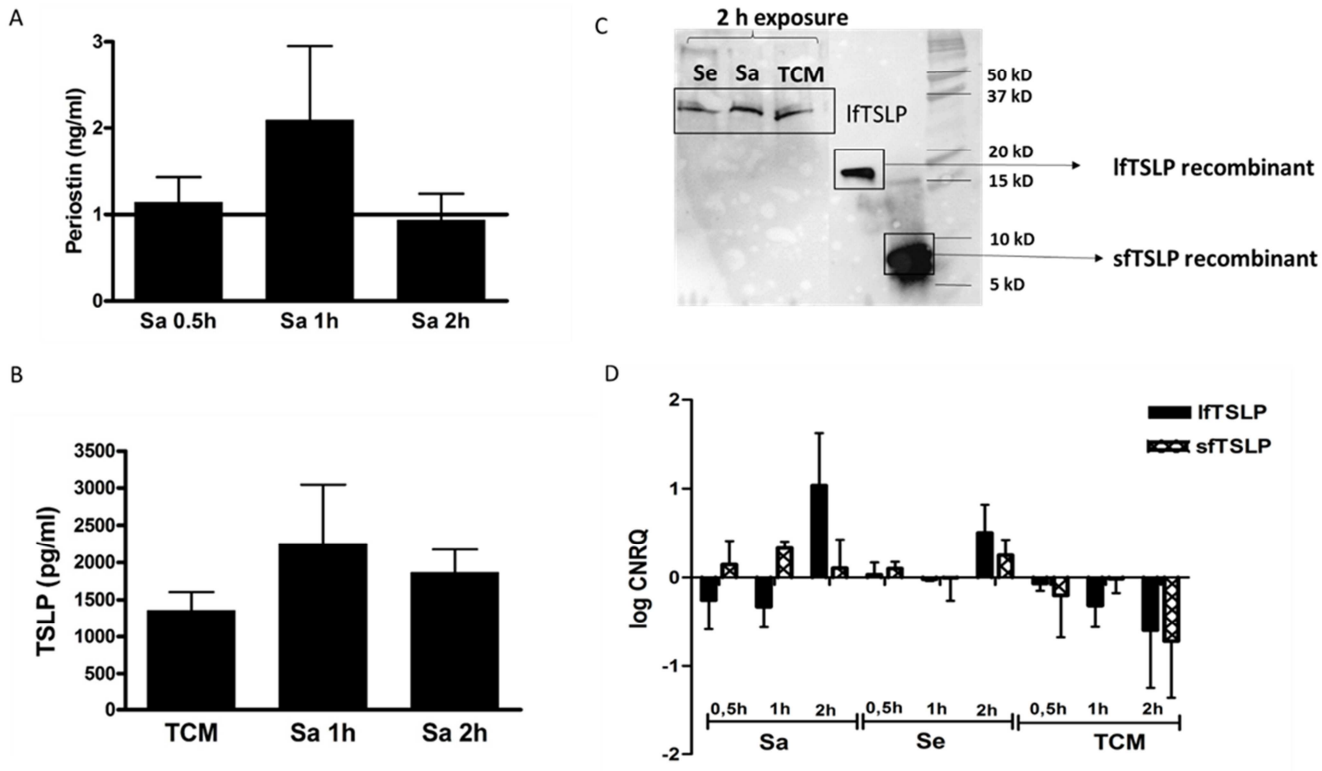


Fig. 7 Periostin and TSLP levels after *in vitro* exposure to *S. aureus*. (A) Periostin levels in tissue homogenates were not significantly elevated after exposure to *S. aureus*. Black line represents the control value (TCM) (n=4). (B) TSLP levels, determined by ELISA, in tissue homogenates were not significantly elevated after exposure to *S. aureus* (n=4). (C) Western blot analysis showing no difference in lfTSLP expression in tissue homogenates after exposure to either *S. aureus*. or *S. epidermidis*. In addition the western blot indicates that sfTSLP was not detected. (D) PCR analysis in tissue homogenates indicates an increase in lfTSLP after 2 hours exposure to *S. aureus* (n=3).

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632

633 **7. Supplemental information**

Primer	sequence
SDHA-FW	TGGGAACAAGAGGGCATCTG
SDHA-Rev	CCACCACTGCATCAAATTCATG
EF1-FW	CTGAACCATCCAGGCCAAAT
EF1-Rev	GCCGTGTGGCAATCCAAT
TSLP short form FW	CGTAAACTTTGCCGCCTATGA
TSLP short term Rev	TTCTTCATTGCCTGAGTAGCATTTAT
long form TSLP - FW	GGGCTGGTGTTAACTTACGACTTCA
long form TSLP - Rev	ACTCGGTACTTTTGGTCCCACTCA

634 **Table S1. PCR primer sequences**

635

Investigated feature		CRSwNP (n/N)	Controls (n/N)
Degranulated eosinophils	< 1% eosinophils/not present	5/15	5/5
	< 10 % eosinophils	6/15	0/5
	< 20 % eosinophils	4/15	0/5
***S. aureus* presence**	Not present	7/15	4/5
	Planktonic < 2 spots	4/15	1/5
	Planktonic > 2 spots no biofilm	1/15	0/5
	Biofilm	3/15	0/5

636 **Table S2. Semi-quantitative evaluation of investigated parameters including degranulation and**

637 **colonization of *S. aureus*.**

638

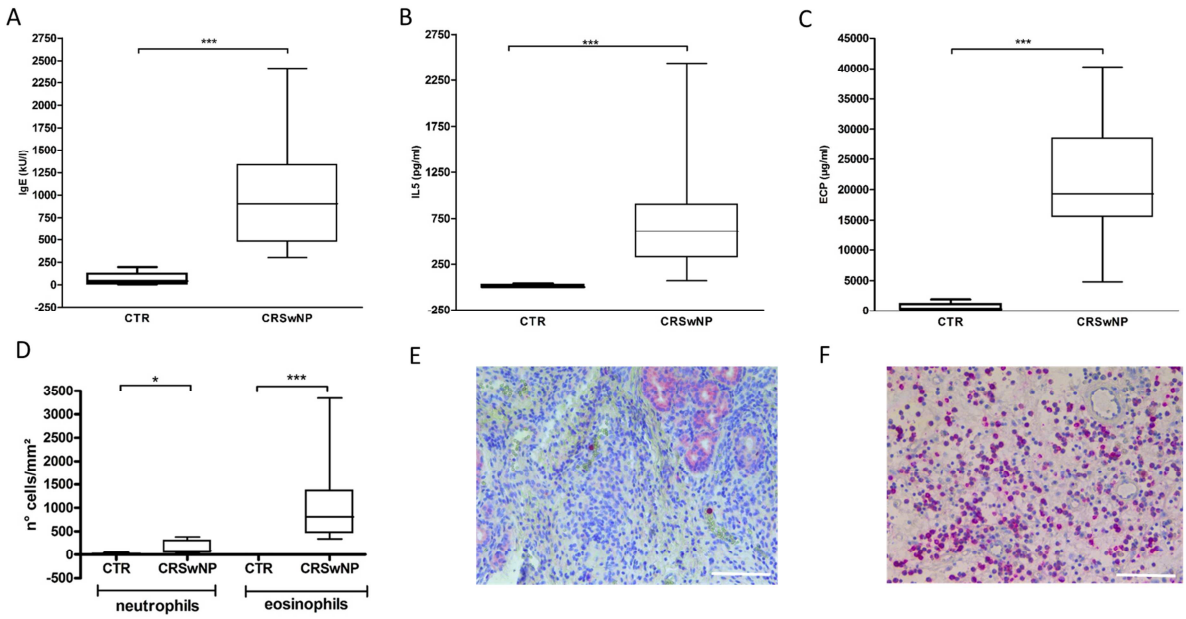
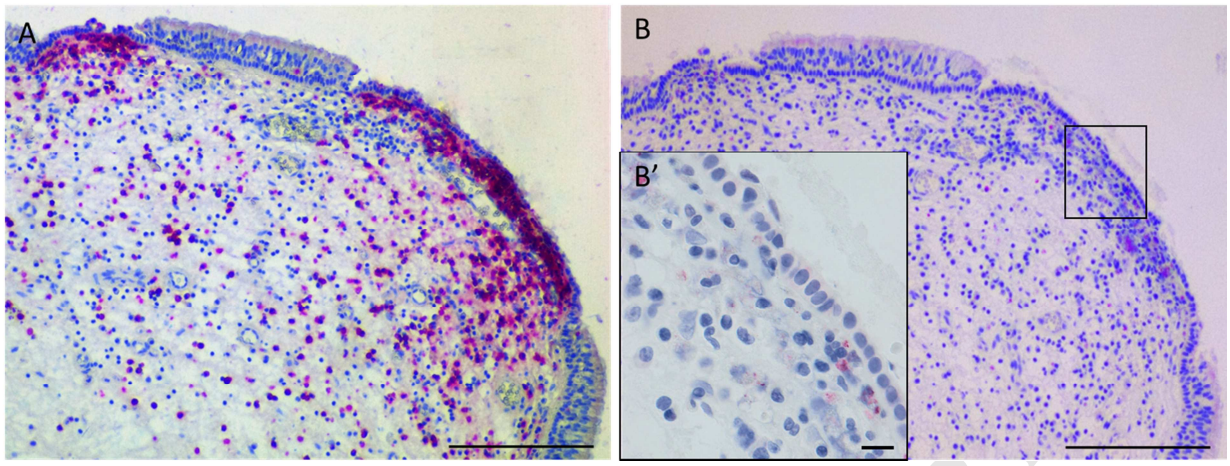


Fig. S1 Characterization of the study population. Tissue levels of IgE (A), IL-5 (B), and ECP (C) were measured in all patients (CRSwNP, n=15) and controls (CTR, n=5) by means of ELISA and were found to be elevated in the CRSwNP patients. Eosinophils and neutrophils (D) were counted in tissue of controls and CRSwNP patients. Tissue slides stained for major basic protein in controls (E) and CRSwNP (F) showing the general distribution of eosinophils throughout the tissue (Scale bar = 100 µm). Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001



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651 **Fig. S2 Subepithelial eosinophils and damaged epithelium.** *Immunohistochemistry was performed*
652 *on subsequent tissue slides and the same region was selected for pictures A-B. The protein of interest*
653 *is stained in red/purple, nuclei are stained with hematoxylin (blue).* (A) An immunohistochemical stain
654 for MBP (red) shows the localization of eosinophils in polyp tissue. (Scale bar = 200 µm); (B) An
655 immunohistochemical stain for caspase-3 (red) demonstrates very few apoptotic cells in the tissue.
656 Inset image (B') shows no evidence for cell lysis. (B: Scale bar = 200 µm, B': scale bar = 10 µm)

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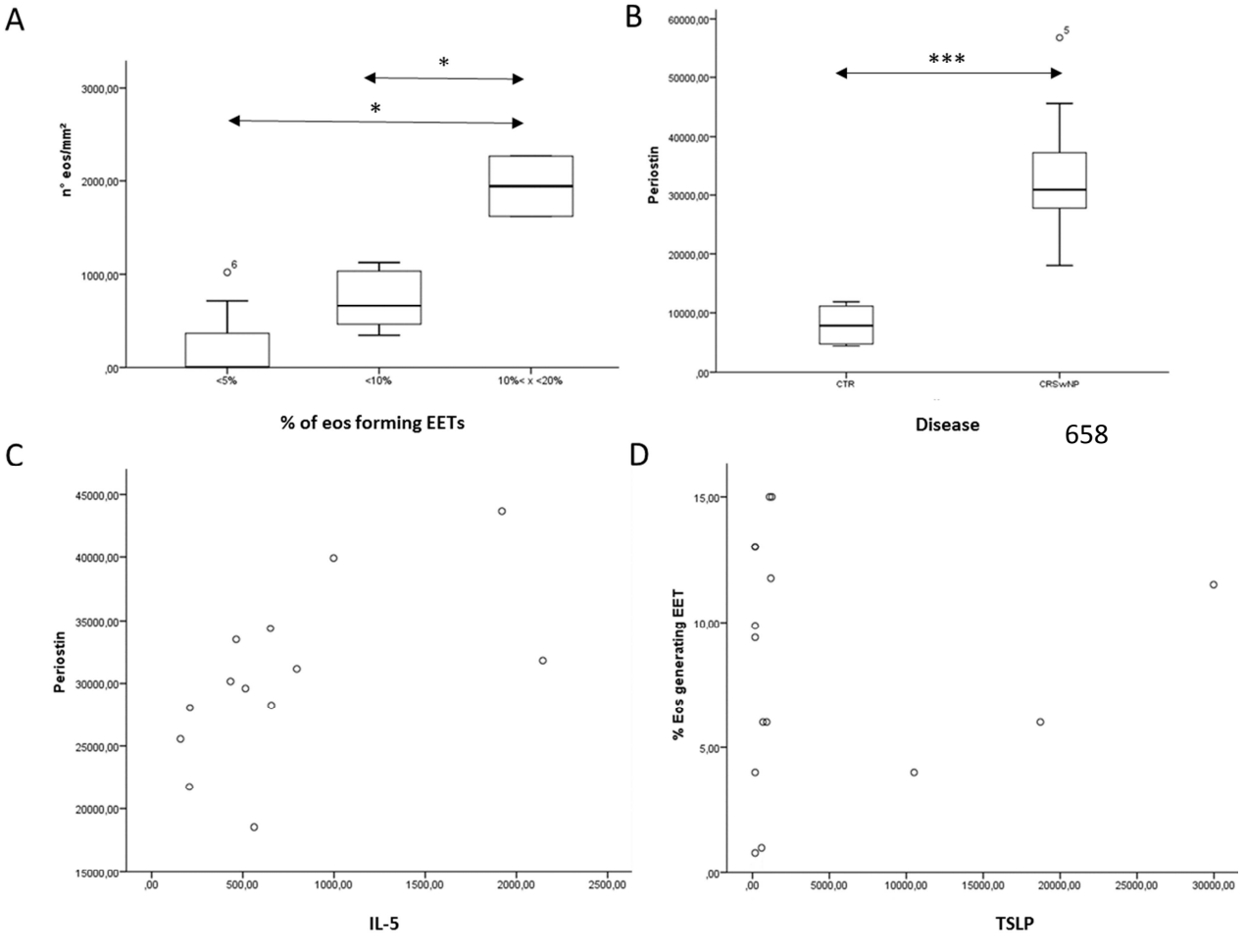

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Fig. S3 (A) Relation between EET formation and number of eosinophils in the tissues. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001 (B) Periostin levels in CRSwNP patients and controls. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001 (C) Scatterplot of periostin and IL-5 levels in CRSwNP patients with a significant correlation (p=0.013, R^2=0.665), as determined by Spearman Correlation. (D) Scatterplot of TSLP and % of eosinophils forming EETs with no correlation as determined by Spearman Correlation.

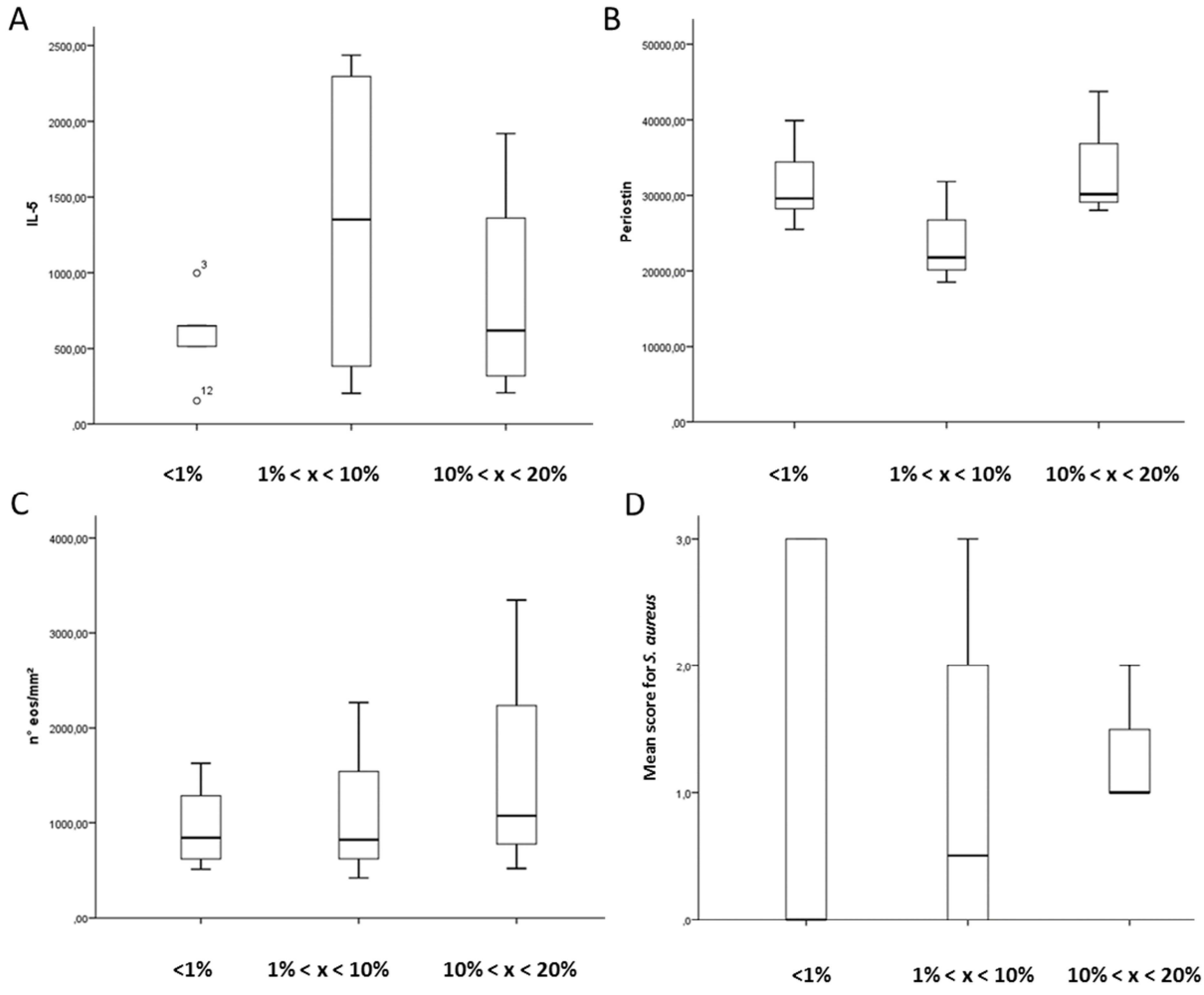

Fig. S4 (A) Relation between IL-5 and percentage of degranulated eosinophils. (B) Relation between periostin and percentage of degranulated eosinophils. (C) Relation between number of eosinophils and percentage of degranulated eosinophils. (D) Relation between *S. aureus* colonization and percentage of degranulated eosinophils.

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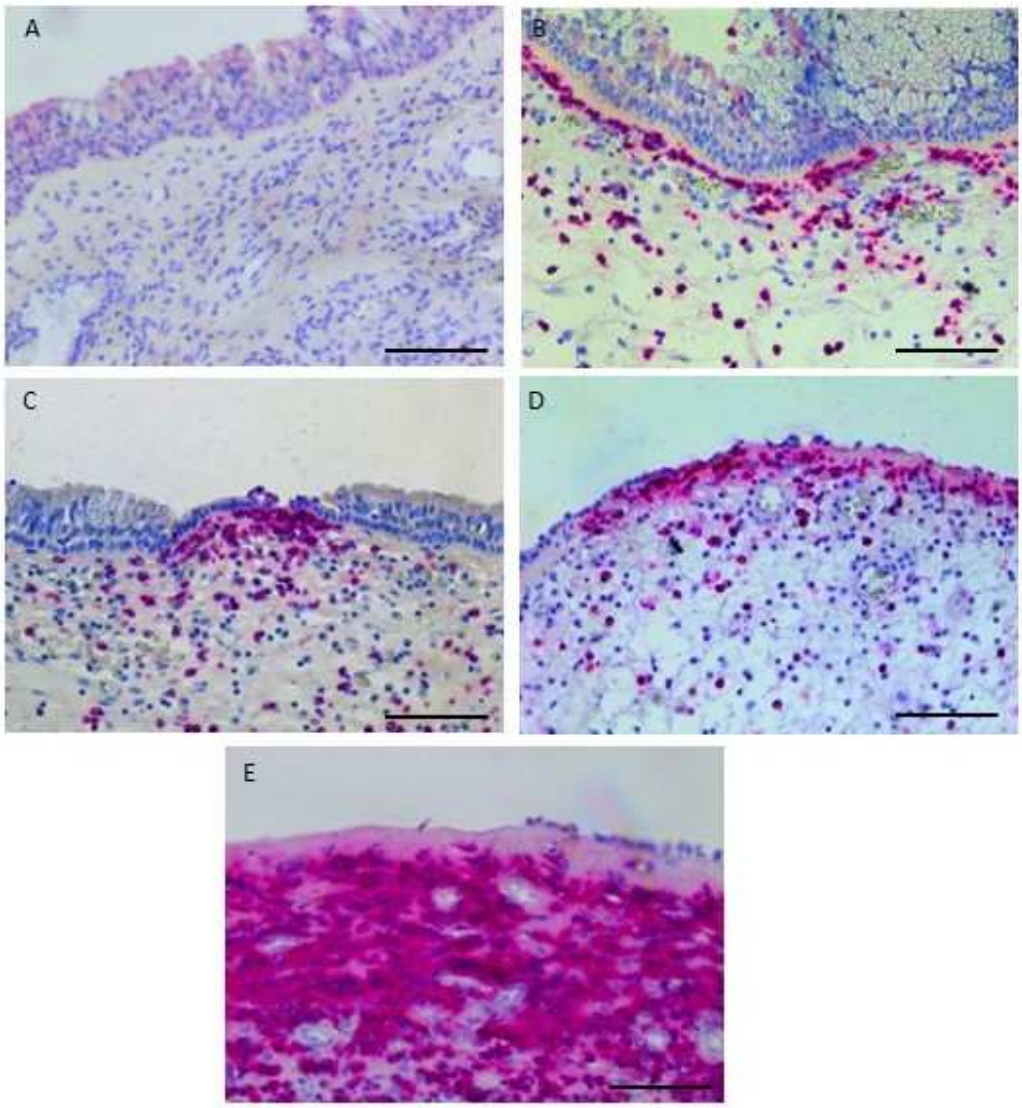
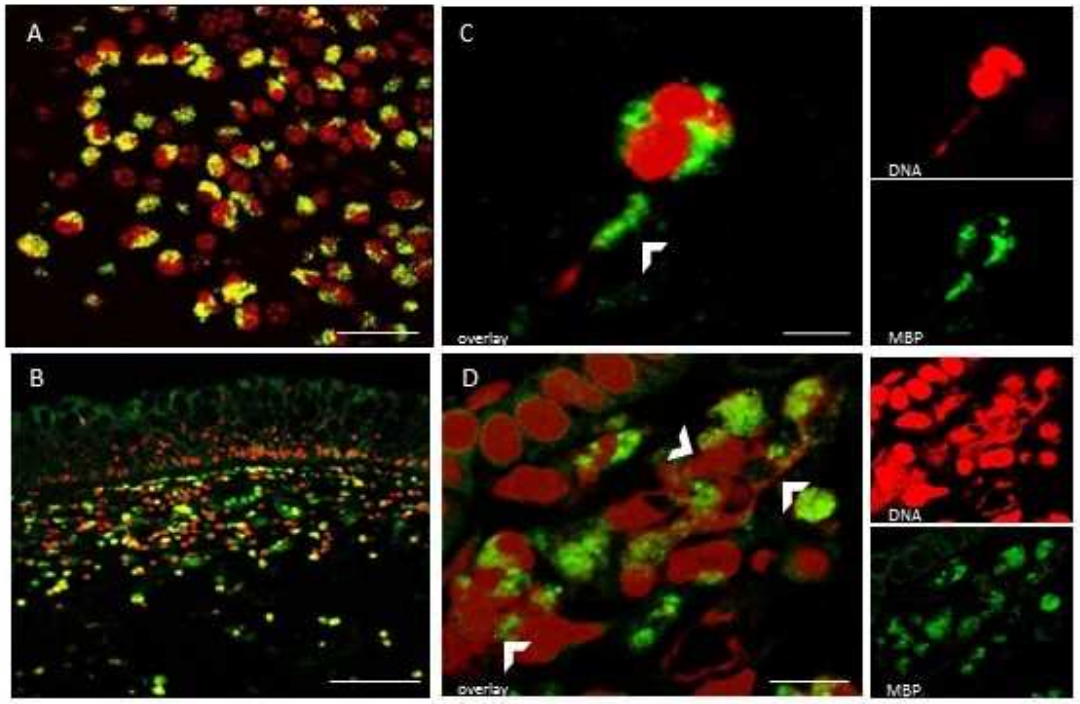
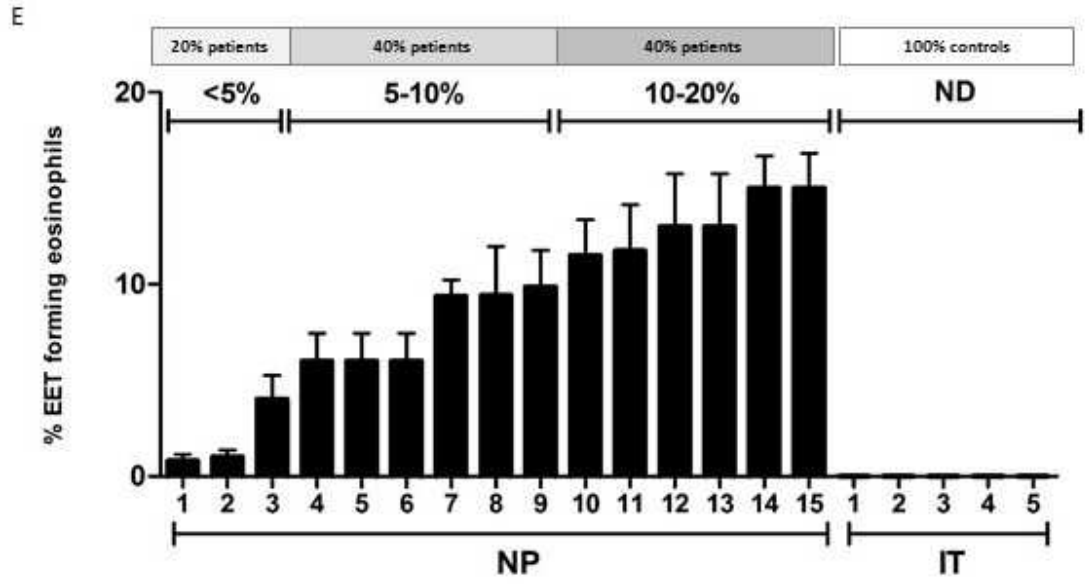
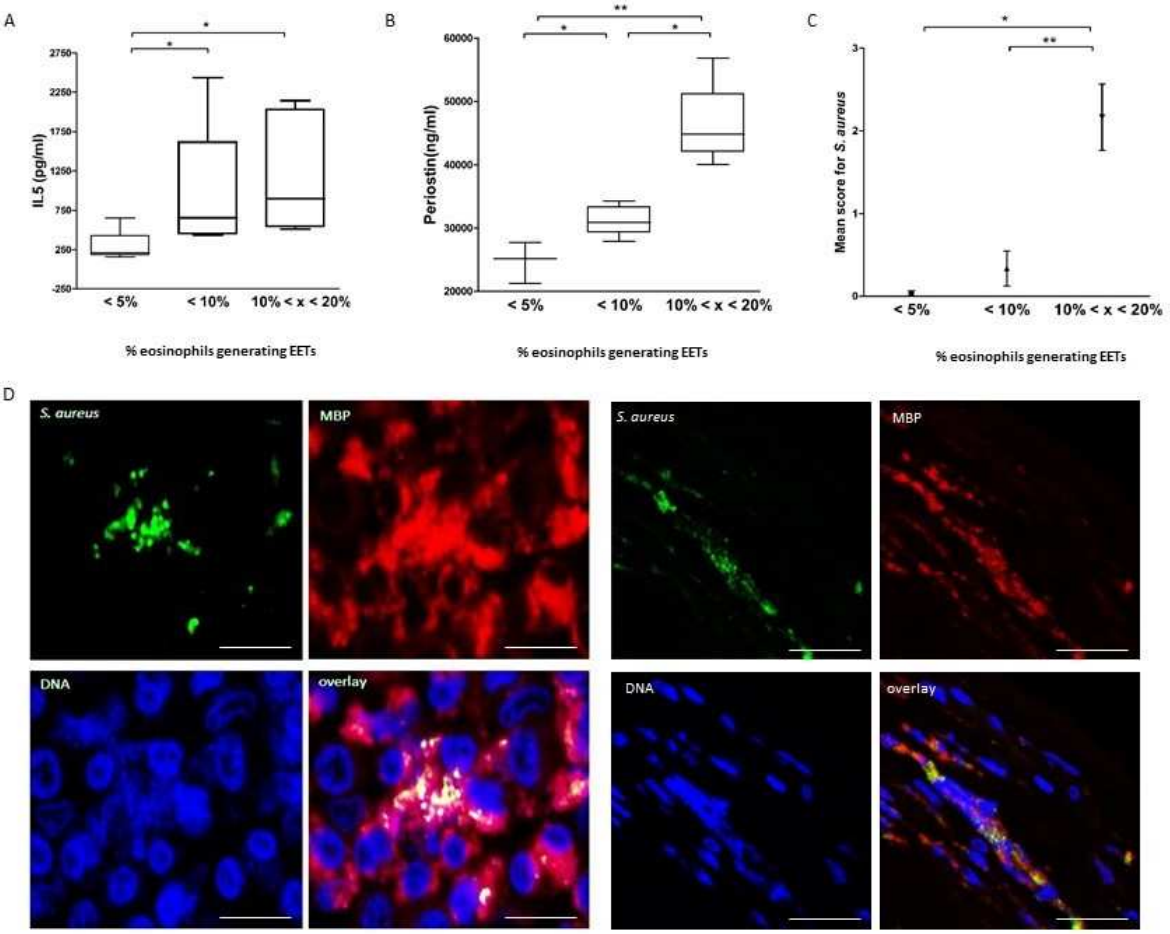
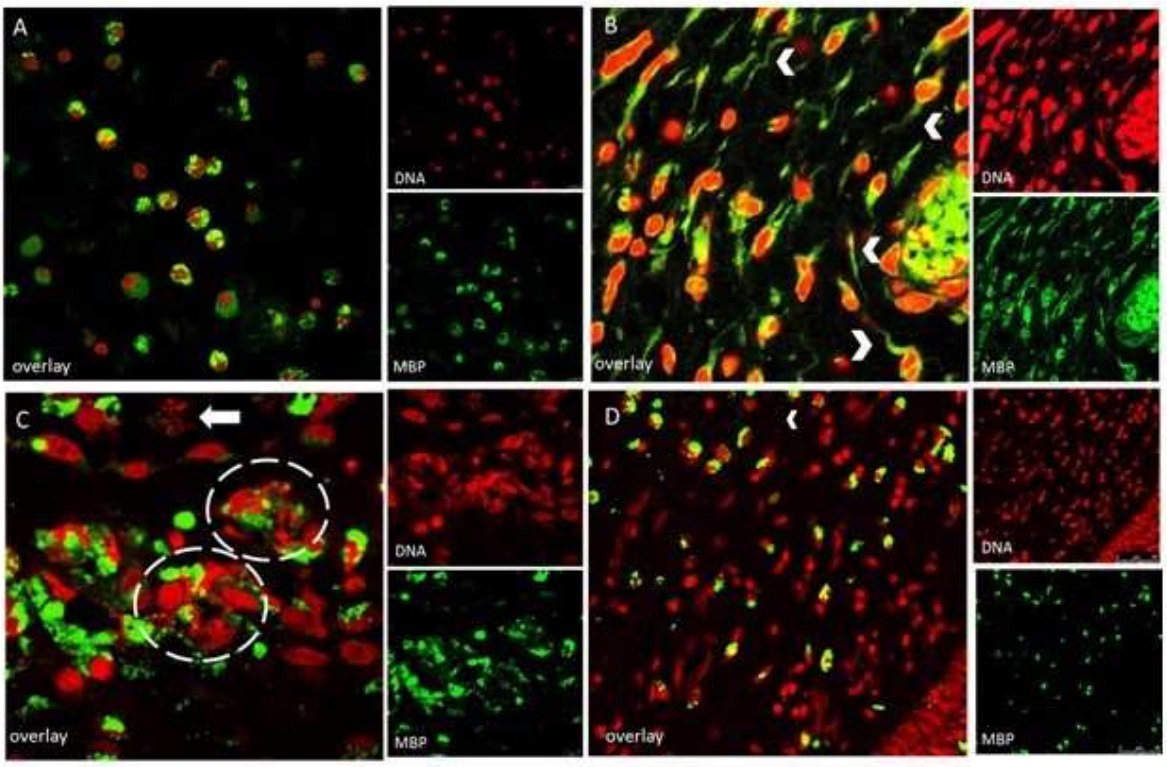
		CRSwNP	Controls
Total cases		15	5
Gender	M/F	10/5	3/2
Age	Median (range)	35 (21-92)	33 (29-42)
Ethnicity		Caucasian	Caucasian
Allergy	-/+	2/12	2/2
	missing cases	n=1	n=1
Asthma	-/+	7/7	1/4
	missing cases	n=1	n=0
Treatment asthma	-/+	4/3	2/2
IgE	Mean ± SD	1448.64 ± 1935.70	62.03 ± 77.95
SAE-IgE	Mean ± SD	6.85 ± 7.41	BDL

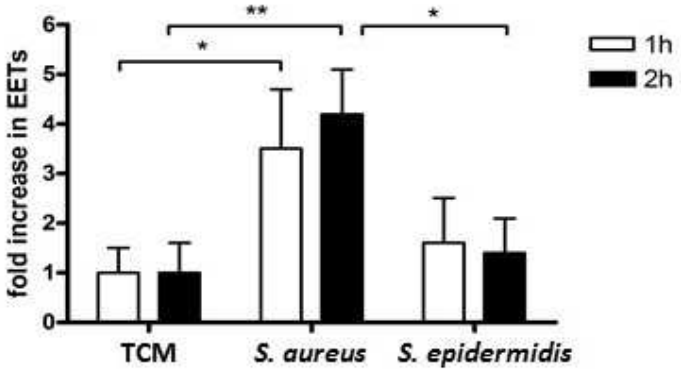


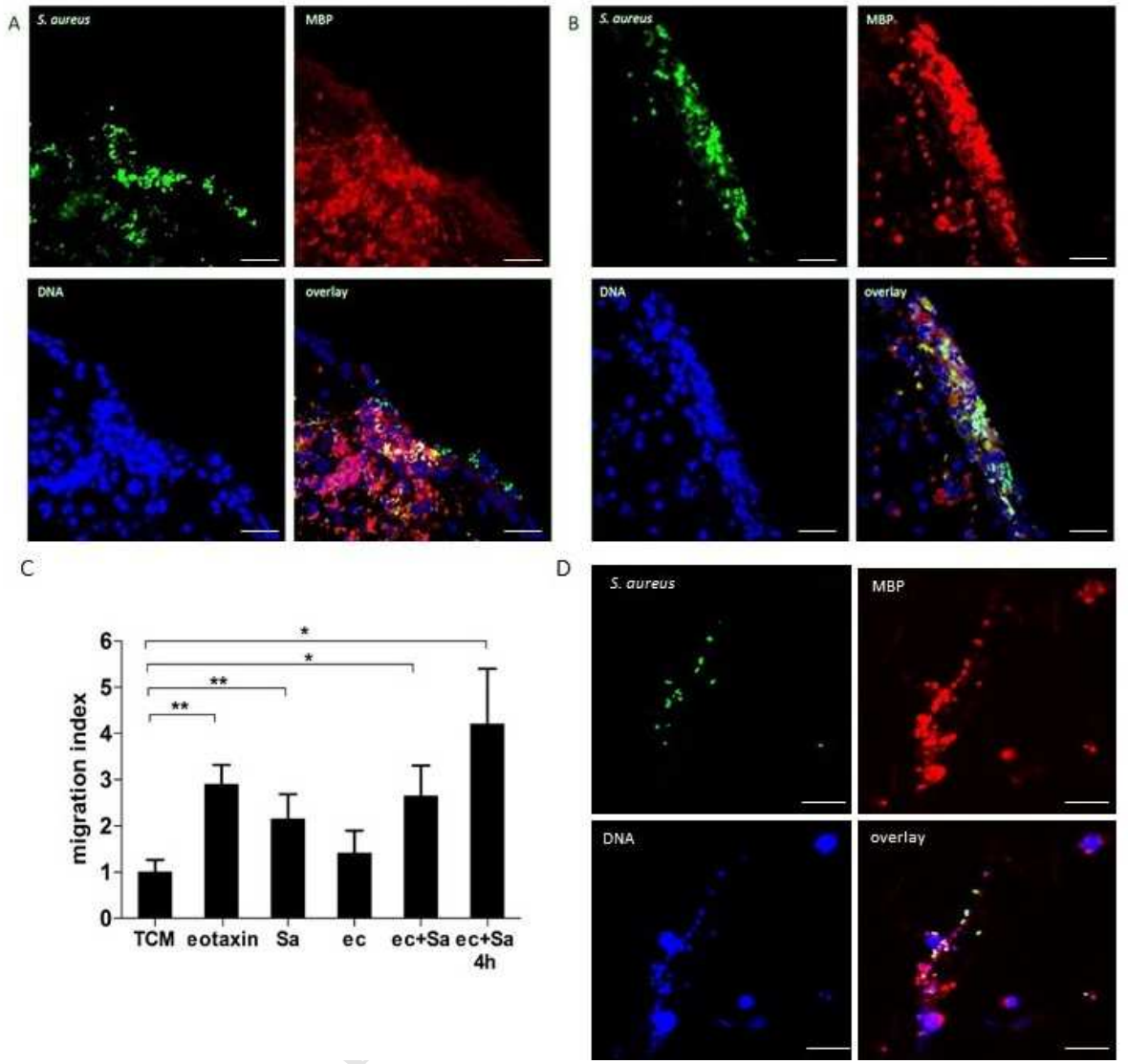

A
B
C
overlay
DNA
MBP
D
overlay
DNA
MBP

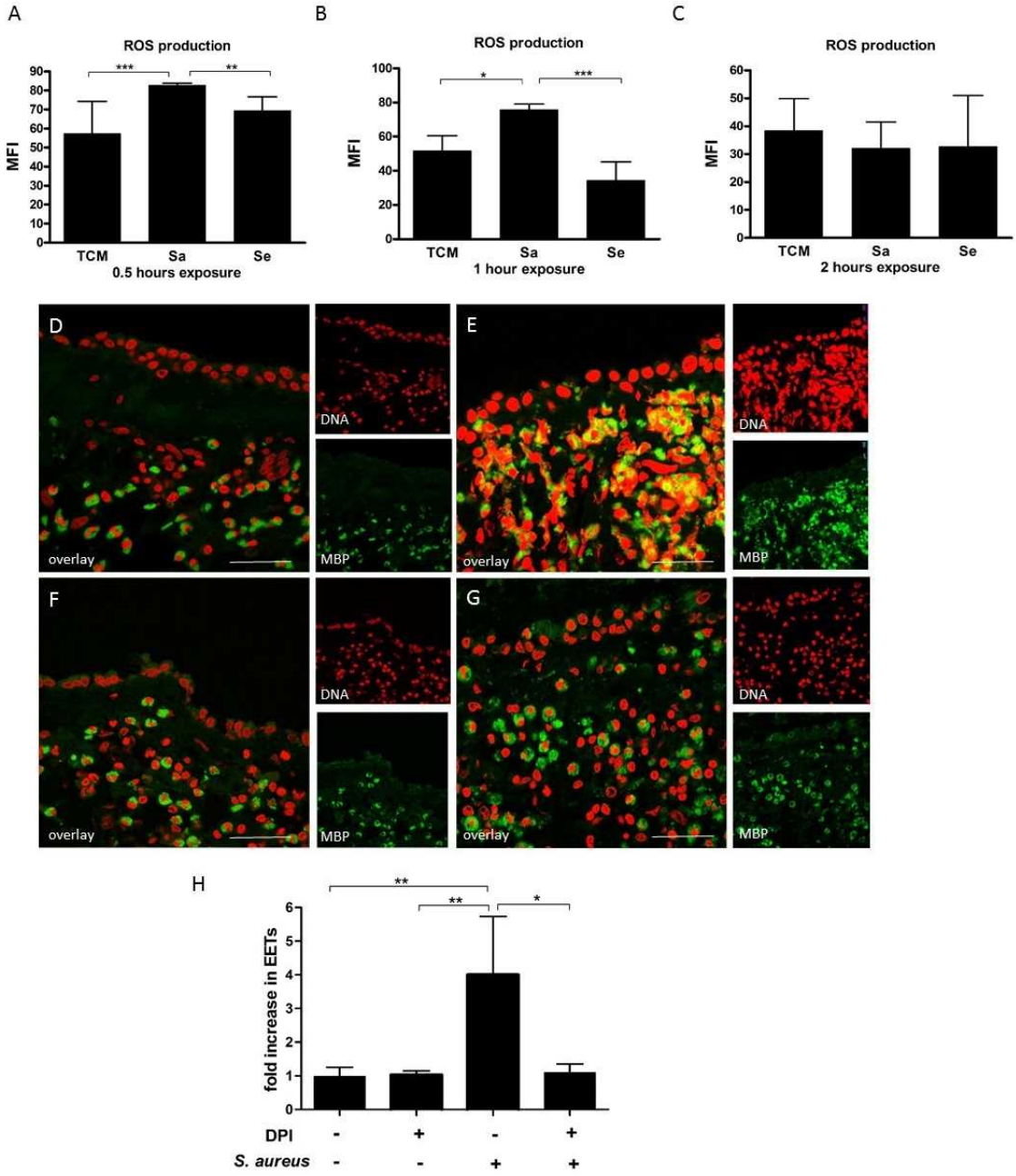

E
20% patients
40% patients
40% patients
100% controls
<5%
5-10%
10-20%
ND
% EET forming eosinophils
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 1 2 3 4 5
NP
IT


A
IL5 (pg/ml)
2750
2250
1750
1250
750
250
-250
< 5%
< 10%
10% < x < 20%
% eosinophils generating EETs
B
Periostin(ng/ml)
60000
50000
40000
30000
20000
< 5%
< 10%
10% < x < 20%
% eosinophils generating EETs
C
Mean score for S. aureus
3
2
1
0
< 5%
< 10%
10% < x < 20%
% eosinophils generating EETs
D
S. aureus
MBP
DNA
overlay
S. aureus
MBP
DNA
overlay


A
overlay
DNA
MBP
B
overlay
DNA
MBP
C
overlay
DNA
MBP
D
overlay
DNA
MBP


fold increase in EETs
0
1
2
3
4
5
6
*
**
*
1h
2h
TCM
S. aureus
S. epidermidis


A
S. aureus
MBP
DNA
overlay
B
S. aureus
MBP
DNA
overlay
C
migration index
0
1
2
3
4
5
6
*
*
**
**
TCM
eotaxin
Sa
ec
ec+Sa
ec+Sa 4h
D
S. aureus
MBP
DNA
overlay


A
ROS production
MFI
TCM
Sa
Se
0.5 hours exposure
B
ROS production
MFI
TCM
Sa
Se
1 hour exposure
C
ROS production
MFI
TCM
Sa
Se
2 hours exposure
D
DNA
overlay
MBP
E
DNA
overlay
MBP
F
DNA
overlay
MBP
G
DNA
overlay
MBP
H
fold increase in EETs
DPI
S. aureus

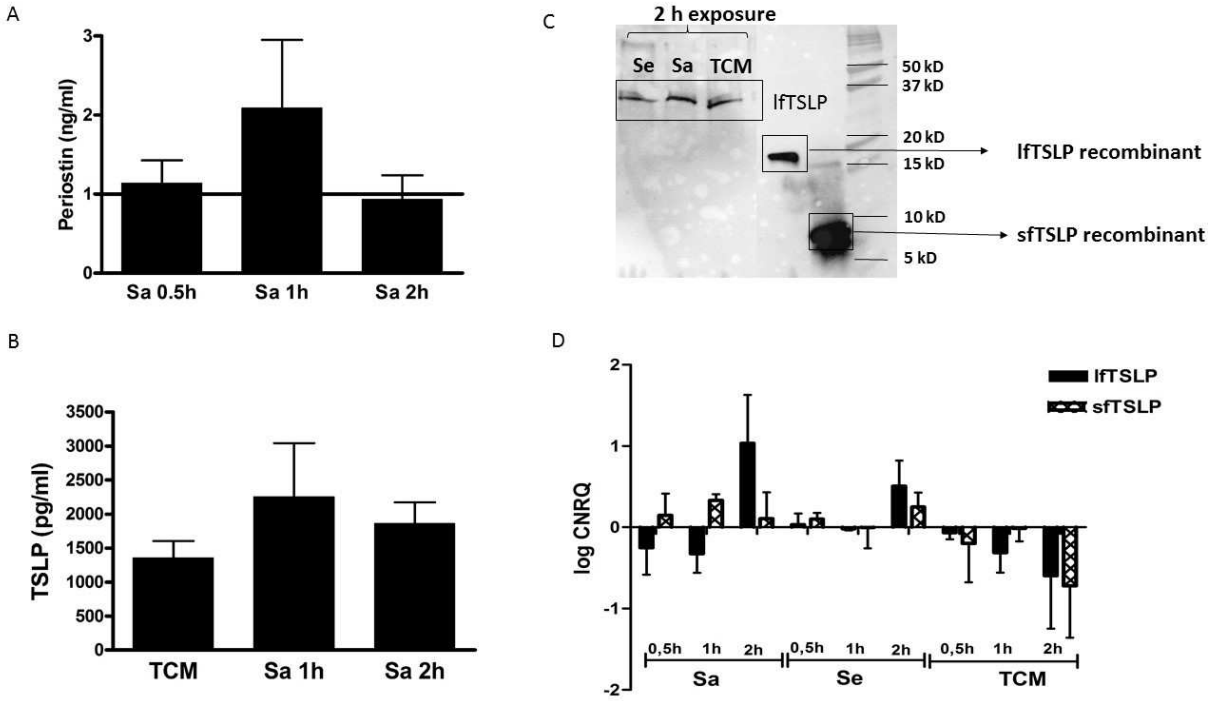


Fig. S1 Characterization of the study population. Tissue levels of IgE (A), IL-5 (B), and ECP (C) were measured in all patients (CRSwNP, n=15) and controls (CTR, n=5) by means of ELISA and were found to be elevated in the CRSwNP patients. Eosinophils and neutrophils (D) were counted in tissue of controls and CRSwNP patients. Tissue slides stained for major basic protein in controls (E) and CRSwNP (F) showing the general distribution of eosinophils throughout the tissue (Scale bar = 100 µm). Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001

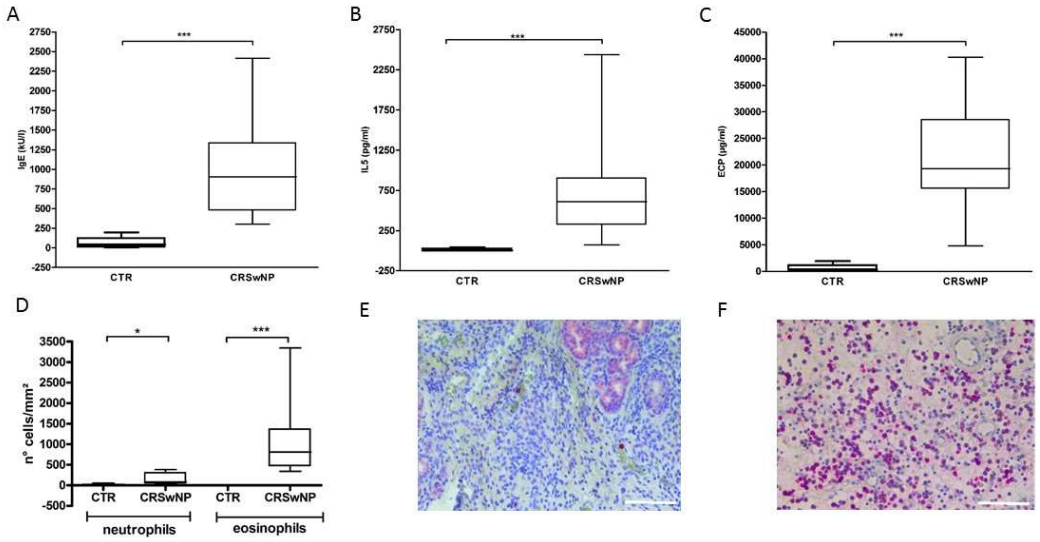
Fig. S2 Subepithelial eosinophils and damaged epithelium. *Immunohistochemistry was performed on subsequent tissue slides and the same region was selected for pictures A-B. The protein of interest is stained in red/purple, nuclei are stained with hematoxylin (blue).* (A) An immunohistochemical stain for MBP (red) shows the localization of eosinophils in polyp tissue. (Scale bar = 200 µm); (B) An immunohistochemical stain for caspase-3 (red) demonstrates very few apoptotic cells in the tissue. Inset image (B') shows no evidence for cell lysis. (B: Scale bar = 200 µm, B': scale bar = 10 µm)

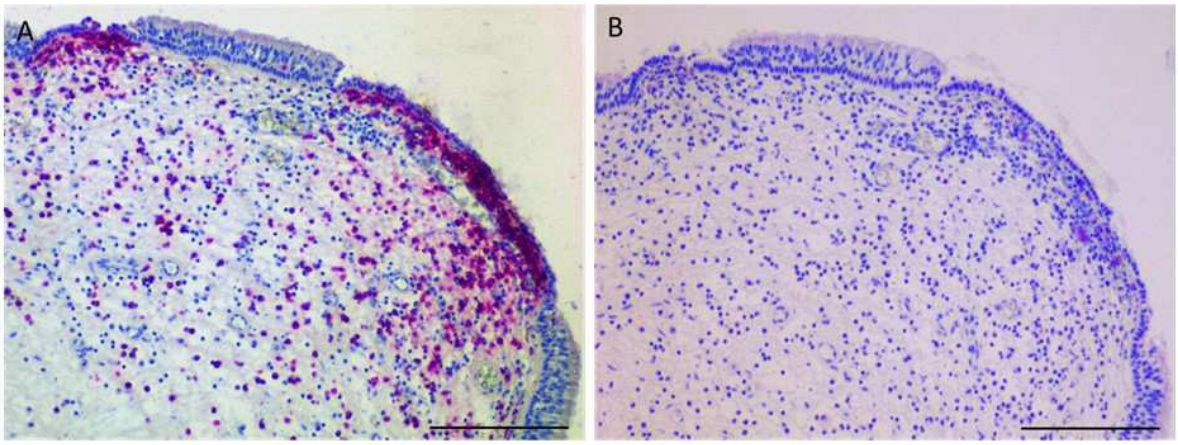
Fig. S3 (A) Relation between EET formation and number of eosinophils in the tissues. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001
(B) Periostin levels in CRSwNP patients and controls. Data were analyzed using a Mann-Whitney U test, significances are expressed as *p<0.05,**p<0.01, ***p<0.001 (C) Scatterplot of periostin and IL-5 levels in CRSwNP patients with a significant correlation (p=0.013, R^2=0.665), as determined by Spearman Correlation. (D) Scatterplot of TSLP and % of eosinophils forming EETs with no correlation as determined by Spearman Correlation.

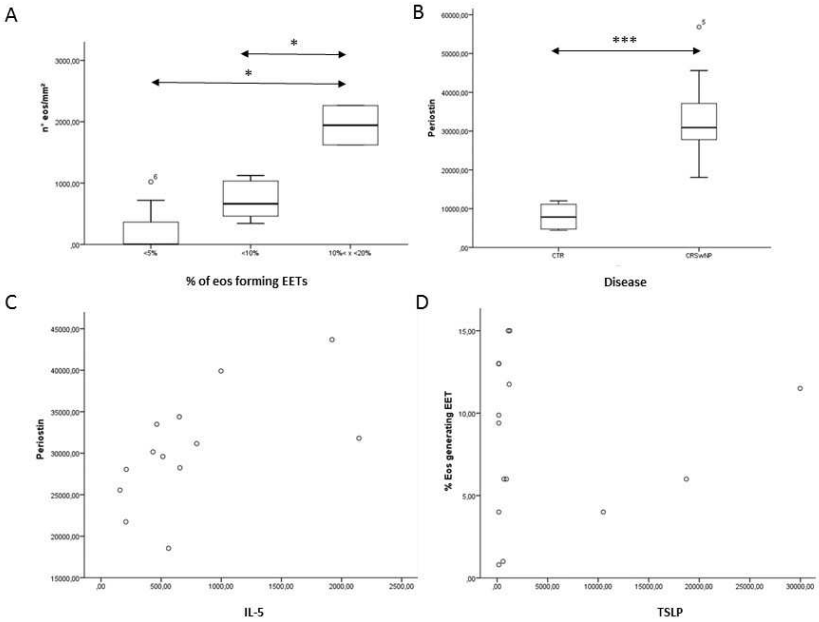
Fig. S4 (A) Relation between IL-5 and percentage of degranulated eosinophils. (B) Relation between periostin and percentage of degranulated eosinophils. (C) Relation between number of eosinophils and percentage of degranulated eosinophils. (D) Relation between *S. aureus* colonization and percentage of degranulated eosinophils.

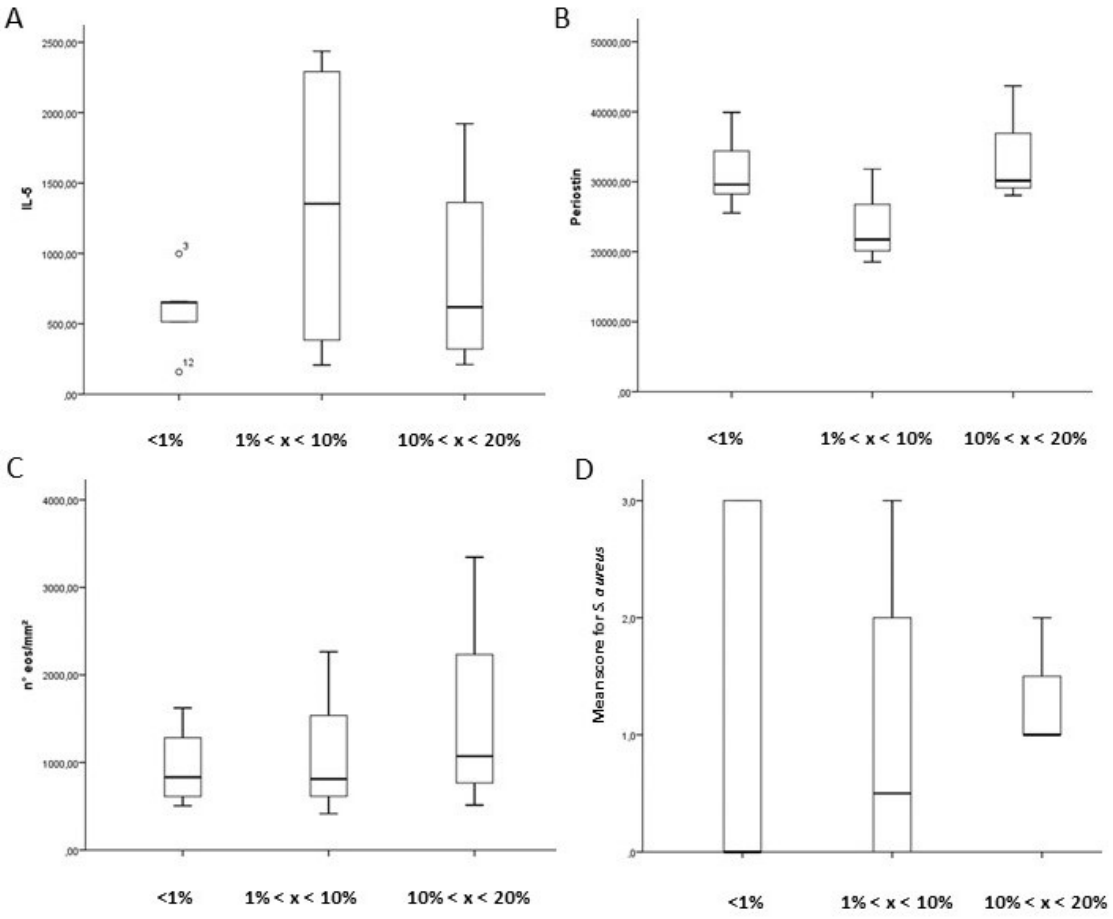
Primer	sequence
SDHA-FW	TGGGAACAAGAGGGCATCTG
SDHA-Rev	CCACCACTGCATCAAATTCATG
EF1-FW	CTGAACCATCCAGGCCAAAT
EF1-Rev	GCCGTGTGGCAATCCAAT
TSLP short form FW	CGTAAACTTTGCCGCCTATGA
TSLP short term Rev	TTCTTCATTGCCTGAGTAGCATTTAT
long form TSLP - FW	GGGCTGGTGTTAACTTACGACTTCA
long form TSLP - Rev	ACTCGGTACTTTTGGTCCCACTCA

Investigated feature		CRSwNP (n/N)	Controls (n/N)
Degranulated eosinophils	< 1% eosinophils/not present	5/15	5/5
	< 10 % eosinophils	6/15	0/5
	< 20 % eosinophils	4/15	0/5
***S. aureus* presence**	Not present	7/15	4/5
	Planktonic < 2 spots	4/15	1/5
	Planktonic > 2 spots no biofilm	1/15	0/5
	Biofilm	3/15	0/5








A
IL-5
2500,00
2000,00
1500,00
1000,00
500,00
,00
3
12
<1%
1% < x < 10%
10% < x < 20%
B
Periostin
50000,00
40000,00
30000,00
20000,00
10000,00
,00
<1%
1% < x < 10%
10% < x < 20%
C
n° eos/mm²
4000,00
3000,00
2000,00
1000,00
,00
<1%
1% < x < 10%
10% < x < 20%
D
Mean score for S. aureus
3,0
2,0
1,0
,0
<1%
1% < x < 10%
10% < x < 20%